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INVESTIGATIONS ON THE NITROGEN NUTRITION OF PLANTS

I. A NEW METHOD FOR THE DETERMINATION OF NITROGEN-REQUIREMENT OF SOILS

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I. *Introduction.* In the determination of fertilisation needs of soils, nitrogen causes the most difficulties, as only a small and very variable part of the total nitrogen in the soil can be used directly by plants. Even for the other basic nutritional elements, potassium and phosphorus, total content in the soil does not always indicate the availability of these elements for plants. Most of the *K* and *P* is not readily available for uptake by plants, but must first be liberated by weathering of soil particles or by the dissolving action of excretions from the plant-roots. It may consequently be looked upon as an equilibrium between the absorbing action of the soil and the dissolving action of the roots.

Regarding nitrogen, however, the situation is fundamentally different. The total stock of this element in the soil is almost entirely restricted to organic substances, either stabilized humus or the still undecomposed particles of vegetable or animal origin. In these organic substances nitrogen occurs in the proteins and in protein-like compounds. By the attack of mineralising microbes, small amounts of this protein nitrogen are continually transformed to inorganic forms — ammonium compounds, nitrites and nitrates. The nitrogen cannot become available for the plants before this disintegration, because the proteins and related compounds cannot be assimilated by the roots. The nitrogen in the soil may thus be divided into two fundamentally different fractions: the bulk of the nitrogen in the unavailable form of organic compounds, and the comparatively much

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smaller part, comprising the mineralised nitrogeneous compounds, all of which can be almost entirely absorbed by the plants immediately. It is true, that of this mineral nitrogen, the ammonium compounds can be absorbed by soil particles, but quantitatively the NO_3-nitrogen in most soils is of much more importance than the NH_4-nitrogen. Besides, the absorbed NH_4-ions are just as readily available for the nutrition of plants and microbes as the nitrogen in solution.

Development of most of the annual crops in the temperate-climate zones starts at a time when the content of mineral nitrogen in the soil is relatively high, since late spring is the period of the highest mineral nitrogen level in the soil as a result of comparatively active mineralisation in the warm days of spring without any appreciable uptake of nitrogen by the very young plants. But this stock of mineral nitrogen can never become very large. During the preceding winter and early spring, the temperature was too low for active disintegration of the humus, and rainfall surpassed evaporation, while the period between improvement in the climatic conditions and the start of plant growth is too short for an accumulation of appreciable amounts of mineralised nitrogen. Numerous reports in the literature as well as our own investigations confirm this observation: it is seldom that more than 20 to 30 mg of mineral nitrogen per kg of dry soil is found in ordinary arable soils at that time of the year. Thus, without an intentional fertilisation, only the first part of the development of a crop can be supported by accumulated nitrogen, while as soon as the crop comes into the stage of its most rapid growth, it starts to absorb so much nitrogen that this absorption surpasses in most soils the production by the mineralising action of microbes. Consequently the stock of mineral nitrogen, available at the beginning of the vegetation-period, is soon exhausted and the crop for the rest of its growth depends exclusively upon the nitrogen liberated by microbes from the humus. This is most pronounced for all types of perennial vegetation — pastures, grasslands, forests and wintercereals. For these even the short period of nitrogen-accumulation in the spring may be levelled off by the early starting growth of the plants.

On the other hand, during the warm summer-season, mineralisation of the nitrogen can become very significant in fertile soils, producing, sometimes within a few weeks, more assimilable nitrogen than the above mentioned stock, accumulated during the spring.

Once this scheme of nitrogen-nutrition of the plants is accepted,

the inevitable conclusion follows, that determination of the amount of any form of nitrogen in the soil cannot give a correct estimate of fertilisation requirements. Only a measurement of the activity and the rate of mineralisation of the nitrogen containing organic matter in the soil, can serve as criterion for this purpose. The limitations of the value of nitrogen determinations become still more apparent when we consider that sometimes even during the summer season the weather-conditions can bring about very rapid and very considerable ups and downs in the nitrogen content. After a period of dry sunny weather, permeating the soil with innumerable cracks, one heavy thunderstorm or a succession of rainy days can deprive the top-soil of nearly all the available mineralised nitrogen.

It is therefore by no means surprising that none of the many proposed methods for the determination of available nitrogen in the soil proved to give correct insight into the fertilisation requirements. It is true, that the determination of the amount of nitrogen, when compared with practical observations of crop development over a long series of years, can give some indications concerning the nitrogen requirements of the soil. Sometimes it is even possible to determine values of nitrogen content corresponding with certain yields of the crops. But these values can never be generalised, since they are valid only for the soil and for the conditions where the study was done. Consequently it was gradually recognised that the value and reliability of such investigations are rather disappointing. G i e s e k e [3]) a.o. when speaking about the determination of the total nitrogen content of the soil, summarised this opinion by saying: .... „Diese Feststellung ist vereinzelt, so das die chemische Bestimmung des Bodenstickstoffs in ihrer Geeignetheit zur Ermittlung des Düngebedürfnisses der Pflanzen als fragwürdig erscheinen muss" ..... And discussing the determination of the mineralised nitrogen of the soil .... „Doch selbst die Bestimmung der löslichen N-Verbindungen ist für die Ermittlung der N-Bedürftigkeit wohl kaum von grosser Bedeutung, da die im Boden vorkommenden Mengen nicht nur sehr klein, sondern auch sehr schwankend infolge der immerwährenden Umsetzungen sind" ..... These opinions became gradually more and more uniformly accepted around 1920, whereby the high expectations of the early days of agricultural research seemed to be destroyed without finding another way to reach the goal.

The first sign of finding a way out in this dead-lock was the intro-

duction of the determination of the nitrogen, available for plants in the soil, simultaneously with an analytical determination of the total nitrogen content of the soil. Through these investigations it was soon recognised that the C : N ratio in the soil was important, and consequently efforts were started to detect the nitrogen requirement of soils from the determination of their C : N ratio in addition to that of their total N-content. But before long it was again realised that the mineralisation of nitrogen depends on more factors than these two alone. Thus conclusions derived from such investigations were still unreliable.

Purely empirical methods for the determination of the availability of nitrogen had no more success: the method of Mitscherlich, the analysis of mature plants or seedlings (method of Neubauer) or the determination of the NO_3-content in plant-juices as proposed by Niklas and Vogel. The first-mentioned method became a failure when applied to nitrogen since the action-coefficient of N is very small. Consequently very much nitrogen had to be used, resulting in severe lodging of the crops. Moreover this determination is very vague as a result of the very rapid changes in the amount of the available mineralised nitrogen. All methods based on the chemical analyses of plants are handicapped by the sometimes very significant surplus-adsorption of nitrogen by plants, giving a complicated and varying relation of the growth to the quantity of absorbed N, so that it proved to be impossible to use thise determinations for a reliable evaluation of the nitrogen requirement of soils.

Better results were unattainable until the first attempts were made to determine the ammonifying and the nitrifying capacities of the soils, especially if these determinations, instead of being made in a liquid medium inoculated with the soil concerned ,were done directly in the soil, with or without (but preferably without) additions of proteins in the first case and ammonium compounds in the second. These ammonification and nitrification determinations were the first step on the right way as they introduced for the first time the principle of keeping the soil samples for a certain period in favourable conditions before analysing for ammonia and nitrate compounds, thereby giving the microflora an opportunity to achieve a certain mineralisation of the humus. One of the first reports about such determinations was made by Bogdanow[1]) as early as 1900, when he described very elaborate and perfected experiments of this

kind. Many years later these methods were improved and standarised still more by N ě m e c [5] [6]), but the best modification of these methods was described by V a r a l l y a y [7]) in 1937.

Yet even these methods did not give the expected clear information. Their first shortcoming is that they concern only one stage in the whole cycle of mineralisation of the humus nitrogen. But the fact that the amount of nitrates and ammonium compounds at the time of sampling is not determined and thus not taken into consideration is much more serious, because this initial concentration of the mineral N proved to be of primary importance in regulating the course of mineralisation, as will be discussed later in detail. Therefore it is even insufficient to determine analytically the concentration of mineralised nitrogen in a soil at the time of sampling for an ammonification and nitrification experiment. Really comparable results can only be obtained when starting on the same very low level of mineral N in the samples. It is even surprising that it was possible to obtain some satisfactory results with the application of such ammonification determinations. This can presumably be explained, considering that very often the sampling is done in summer, when most soils are practically exhausted by the crops and have very small quantities of mineral N. In rather poor soils such periods of very low levels of mineral N may occupy practically the whole year, since in such soils only twice a year, in late spring and early autumn the mineral N-content is increased. Generally speaking, these ammonification and nitrification experiments cannot claim to be consistent and correct applications of the sound principle that the nitrogen requirement of a soil can only be determined when the whole course of mineralisation is followed, starting on the level of a very low concentration of mineralised nitrogen.

In the subjoined a new method will be described, based on the same ideas but trying to follow them as consistently as possible.

II. *The principle of the method.* More than 10 years ago, performing ammonification and nitrification determinations in the soils of the recently reclaimed Wieringermeer-polder, it was observed, that the results of these determinations were greatly influenced by soil conditions at the moment of sampling. Comparable and reproducible results could only be obtained when the sampling was restricted to times of similar conditions and with the same level of mineralised

nitrogen in the soils. In all soils, but especially in those with a low humus content, increase in mineralised nitrogen proved to be the more rapid, the lower this amount was at the moment of sampling. The next step was consequently to try to reduce the concentration of mineralised nitrogen artificially, if too high, before starting the determination, by extracting the mineralised nitrogen from the soils. By this treatment the soil samples were put under the conditions prevailing in the soils during most of the year. It now proved possible to get good results also when the amount of mineralised nitrogen in the soil happened to be high when the samples were taken. These results were then comparable with those obtained in periods of very low concentration of mineralised nitrogen. The best results could be obtained, when this extraction was performed not by percolation of the samples by water but by cultivation of a fast growing crop to absorb the N from the samples. Hereby the soil structure was altered less than by the water treatment.

These fundamental observations were sufficient to indicate the right way for the development of a standardised, generally applicable method for determining the intensity of mineralisation of nitrogenous compounds in humus, and indirectly also of the nitrogen requirements of soils. This method comprises the following successive steps: 1. Extraction of available mineralised nitrogen, preferably by placing the samples in shallow containers and sowing them thickly with a fast growing crop. 2. Removal of the crop and transferring of the samples to conditions ideal for mineralisation of the humus. 3. Analytical determinations at regular intervals of the content of mineralised nitrogen in the samples kept under these favourable conditions. 4. Plotting the results in graphs. The course of the gradual increase in mineralised nitrogen, plotted against time, then offers a measurement of the intensity of mineralisation.

Fundamentally this method can be divided into two periods: the *extraction-period* followed by the *period of regeneration* of the stoc of mineralised nitrogen removed by the extraction. During the regeneration period the samples must be kept under very constant conditions, accurately standardised, to assure sufficiently comparable results.

It must be emphasized that, in this method, the maximal amount of mineralised nitrogen finally reached is not an indication of availability of nitrogen for plants, but rather the rate of increase of this

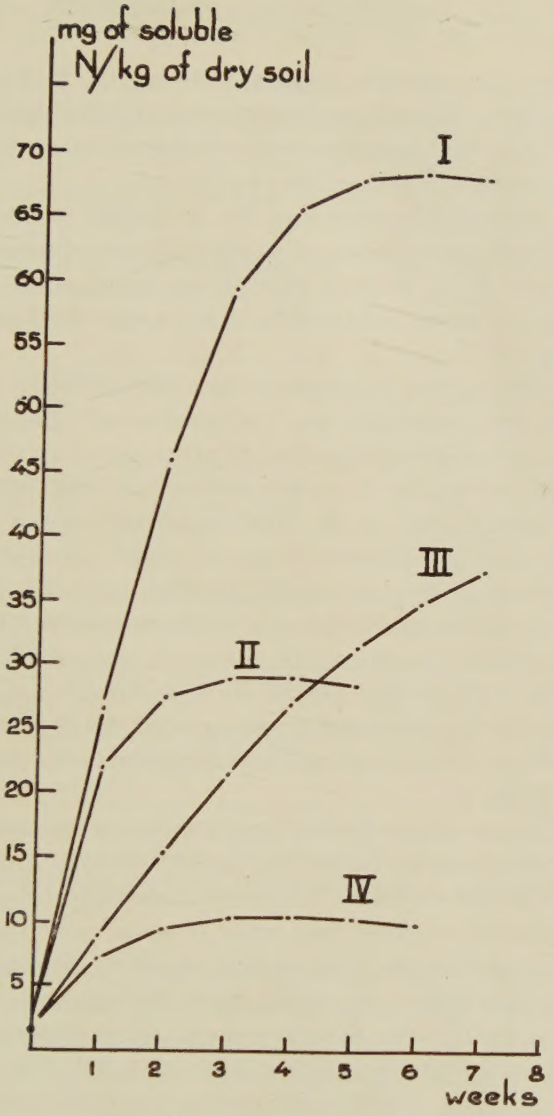

Fig. 1. Diagrammatical, "ideal" curves, indicating the mineralisation of nitrogen.

I: Soil ensuring a very rich nitrogen-nutrition of the plants. A curve with a very steep slope, running up to a high level.

II. Soil giving a moderately good nitrogen-nutrition. A curve with a steep slope, but bending over at a low level within two weeks.

III: Soil giving a poor nitrogen-nutrition. A flat curve, but running up to a comparatively high level. Under favorable conditions a rather high level of mineralised nitrogen can be formed.

IV: Soil with a very poor nitrogen-nutrition. A flat curve bending over to the horizontal within two weeks at a very low level.

Soil II supplies the plants decidedly better by nitrogen than soil III, since the slope of it is much steeper during the first few weeks.

amount during regeneration, especially during the first part of the regeneration. It is this rate and not the final level of mineralised N which represents the quantity of absorbable nitrogen, provided to the growing plants in a unit of time by the soil.

The best method of assembling the analytical results obtained from repeated determinations of soluble nitrogen during regeneration is without doubt to plot them in a graph against time. The possible types of curves obtained by doing so are diagrammatically shown in Fig. 1.

Apart from the principle on which the whole method is based, but nevertheless very important, was the question whether the level of the whole mineralised nitrogen can be represented by the determination of only one part of it — the nitrates. It is true that the nitrates in most soils are by far the most important constituent of the whole complex of the different forms of soluble nitrogen, and also that they belong to the most readily absorbed part of it, but a more detailed study of this problem led us to the conclusion, that even in soils under ordinary conditions, the ammonia compounds and sometimes also the water-soluble organic nitrogenous compounds (amino acids, urea and others) may not be disregarded. An illustration of the figures on which this conclusion is based is given in the data brought together in Table I.

These figures are selected from a large number of similar determinations and they can be backed by analogous data from different publications in the extensive literature concerning this subject, but they are sufficient to show that while in most cases the NH_4 and organic components in the total amount of soluble nitrogen are really very small, sometimes, especially when dealing with permanent grasslands or peaty soils, they are much larger, representing such high percentages that they cannot be disregarded. The determination of the nitrogen available for plants, based on analysis of the nitrates only shall therefore always be a rather uncertain method. Moreover, the process of nitrification and the last phases of disintegration of the soluble organic compounds are very fast, consequently the ammonia compounds and the soluble organic nitrogen may be considered as equivalent to the nitrates for the nutrition of plants, independent of the question whether the NO_3-ion really is the most suitable form of nitrogen-nutrition for the plants.

Both these considerations resulted in the decision not to rely upon

TABLE I

Different forms of nitrogen found in clear water-extracts of some representative soils, expressed as mg N per kg of dry soil

Characteristics of the soils (sampled from 0–20 cm deep)	Date of sampling	Nitrogen determined as	
		NO_3	NH_4
1. Heavy clay soil from the Groetpolder, province of North-Holland, arable land, rich in nitrogen, lying fallow	7- 7-1941	20.4	1.3
	4- 8-1941	21.1	0.2
	1- 9-1941	10.7	0.0
	8-12-1941	7.6	0.3
2. The same soil but in clover	7- 7-1941	11.1	0.9
	4- 8-1941	10.6	0.8
	1- 9-1941	17.9	0.4
	8-12-1941	38.0	2.1
3. Light sandy clay near St. Anna-Parochie, province of Friesland, arable land, lying fallow	7- 7-1941	20.3	0.7
	2- 9-1941	11.0	0.3
4. The same soil but in clover	7- 7-1941	8.0	0.2
	2- 9-1941	6.3	0.3
5. Heavy clay-soil in the Province of North-Holland, permanent grassland, that year used for hay-making	29- 7-1942	5.0	2.8
6. Approximately the same soil, permanently used as pasture	4- 8-1941	11.0	2.0
	24- 8-1942	5.6	5.0
7. Boggy soil, slightly acid, very rich in organic matter, near Giethoorn, Province of Overyssel arable land, in potatoes [1])	15- 7-1946	14.3	24.0

[1]) While in the other samples inlcuded in this table NO_3 and NH_4 account for nearly all the water-soluble nitrogen, leaving only negligible quantities for other nitrogenous compounds, in this boggy soil about 3 mg N was found as unidentified nitrogenous compounds.

the determination of nitrate-nitrogen only but to apply a method giving the total nitrogen soluble in water, thus representing the nitrogen immediately available to the plants. In the beginning the Jodlbaur-modification of the Kjeldahl-method was used, but in searching for a quicker and easier method, it was soon recognised that the different modifications of the method of reducing all nitrogen-forms in an alkaline medium by nascent hydrogen were able to give very exact and reproducible results, identical with those of the Jodlbaur-method when checked with the latter. It was also possible to recover quantitatively known amounts of added pure nitrates, ammonia-compounds, urea and amino acids. The hydrogen is evolved by boiling alkali reacting on finaly ground metal alloys. The advantage of these methods is the simultaneous destruction, reduc-

tion and distillation, which makes this method much quicker. From the numerous possible modifications, that using 3% NaOH and the Devarda-alloy was selected in a slight modification. This method proved to be very reliable and, when standardised, to allow very rapid serial work, therefore it was used almost exclusively throughout this whole investigation.

III. *The method.* The taking of the samples does not need to be described here, since no special manipulations are required. Only the usual rules for all similar samplings must be observed. But within these limitations the whole procedure must be improved and refined as much as possible, since the critical factor determining the rate of nitrogen-regeneration is humus, which is an insoluble substance, very irregularly distributed throughout the soil. Therefore sampling and mixing of the samples have to be done with the greatest possible care and the samples must be composed of a sufficiently large number of borings [4]). In most cases the samples comprised 25 or 50 borings, regularly distributed over the entire surface of the plot under investigation. The samples consequently were rather large, sometimes exceding 10 kg since it was impossible to use borers with a small diameter on the wet and sticky soils.

The question, how deep must the samples be taken, cannot be answered here, since that depends on the purpose of the investigation. On regularly ploughed arable land it is advisable to take samples from the entire turned layer. The surface-layer of 2 to 3 cm is often in an unusual condition — it may be crusty or very dry or crumbled. It may therefore be advisable to remove this layer before sampling. On grassland with a tough sod it is immerative that the turf be removed since it would otherwise greatly increase the amount of fresh organic matter in the sample. The disintegration of this fresh organic matter, generally not rich in nitrogen, should appreciably decrease the rate of nitrogen-mineralisation. Even much smaller quantities of such fresh organic matter (especially living thin roots of plants) sometimes causes very confusing deviations from the normal results.

In the mixing and pretreatment of the samples before analyses, it proved to be impossible to mix the large primary samples by a simple moulding by hands or scoops, especially when the soil was wet and sticky or contained stiff lumps. Yet this mixing

had to be done with much care in fiew of the irregular distribution of the humus in the soil [4]). Consequently all the large heavy samples had to be taken home for mixing and reducing to the required size by special crumbling and distributing apparatuses. The apparatus for crumbling the samples was designed and built especially for this purpose as a rather rough but strong blacksmith construction. It is diagrammatically shown in Fig. 2.

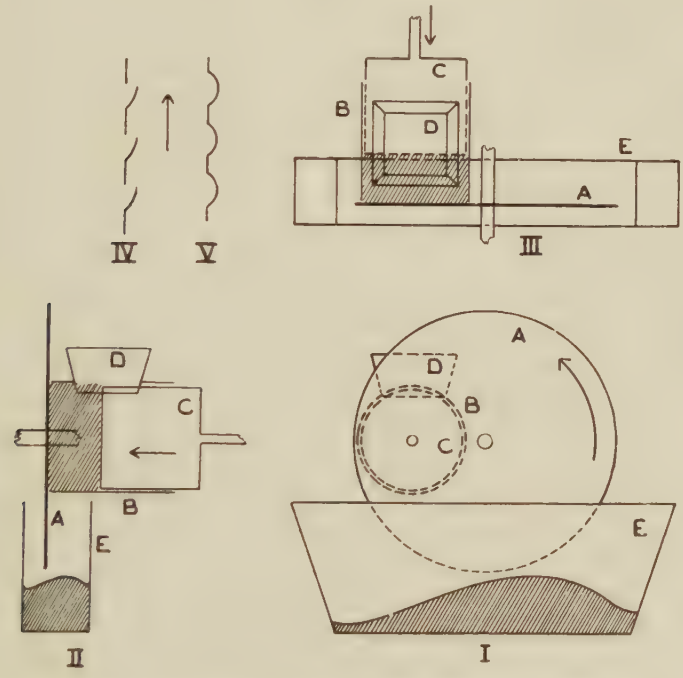

Fig. 2. Digaram of the apparatus for crumbling the soil samples.
I: Side-view.
II: Vertical cross-section.
III: Plan.
IV: Radial cross-section of the disk on a larger scale.
V: Tangential cross-section of the disk on a larger scale.
The lever for moving the piston and the handle for revolving the disk are not shown in the diagram.

The most essential part of this apparatus is a strong round iron disk revolving in a vertical position (*A*). The disk is perforated by a large number of notches of the same type as in a rough soap-grater (See Fig. 2 IV and V). It revolves in front of and very near the outlet of a horizontal, hollow

cylinder (*B*). In this cylinder a piston (*C*) can be moved by a lever. Through a funnel-shaped opening on the upper side of the cylinder (*D*) the soil can be poured into it. Then simultaneously the piston is pushed in by the lever and the disk is revolved. Hereby the soil is pressed against the disk and the notches scrape thin shavings from the soil. These shavings are then collected in a trough (*E*) underneath the disk. It was possible in a short time to transform even the stickiest and thougest soils to a crumbling mass.

From the crumbled primary sample, small samples can now be taken for the determination of moisture and mineralised nitrogen content at the beginning of the whole procedure. The most adequate method for this secondary sampling is the use of a so called "sectorial divider" [4]), which produces a completely reliable sampling when the material is sufficiently finely crumbled.

The sample is now poured into a shallow container and densely sown with a fast growing crop, which absorbs much nitrogen in a relatively short time. The best results could be reached using ordinary spinach (*Spinacia oleracea* L.). As containers, the shallow unglazed earthenware dishes used by horticulturists for nursing of seedlings proved to be adequate. The depth of these dishes is only about 8 or 9 cm; thus while spread out in a comparatively shallow layer, the sample is quickly extracted but it suffers from very rapid evaporation. It is thus necessary to restore the original water content frequently by watering. Since the dishes are made from porous unglazed earthenware, the formation of waterlogged anaërobic corners in them is unlikely. These dishes should not be deeper than 10 cm because the depth of the soil may not exceed about 8 cm, and high edges above the soil would cause shadow-strips and weak growth of the spinach. The minimum size of the dishes must be just sufficient to contain enough soil for the whole series of analyses. Since in our investigation all determinations of mineralised nitrogen were made as macro-analyses, each determination required 300 g while a complete series comprised 6 or 7 determinations. Thus the samples had to be at least 2.5 kg. But to prevent abnormal conditions in the last sub-samplings, the minimum weight of the samples had to be fixed at 5 or 6 kg. Very adequate proved to be the size of these earthenware dishes with a capacity of about 7 kg of soil. However, during the war and the first post-war years it was impossible to get such earthenware dishes. They had to be replaced by small wooden cases of approximately the same shape — a very bad

substitute. Under the conditions of regeneration of the mineral nitrogen in the thermostate, high temperature and moisture caused a very rapid decay of the wood by wood-rotting organisms, and the growth of these organisms was only possible when part of the mineralised nitrogen released in the soil was absorbed by them. The result was thus a delayed and disturbed mineralisation. This shortcoming of the wooden cases became more and more apparent when they were used for a long time. Therefore each case could be used only 2 or 3 times, before the results became too much affected by the decay of the wood. At the laboratory of Dr. C r o w t h e r at the Rothamsted Agricultural Experimental Station, round dishes of glass are used for a similar purpose (unpublished oral information). No difficulties resulting from anaerobic conditions near the bottom have been observed at Rothamsted in these impermeable containers. It may therefore prove to be possible to alter the whole procedure to micro or semi-micro analyses and consequently also to much smaller samples and containers. But micro analyses require more carefully mixed and homogenised samples. It will then therefore be necessary to change and to improve the crumbling and mixing of the samples.

To ensure a quick and regular germination of the spinach, the seed must be distributed very regularly over the whole surface and must then be covered by a layer of the soil approximately 1 cm thick. When earthenware dishes of approximately 30 × 30 cm are used, the best quantity of good germinating seed for one dish is about 700 grains of seed (approximately 15 g).

During the summer season the light-intensity is sufficient for raising healthy spinach-crops in a hothouse; only the usual measures of ventilation, prevention of burning during the hot hours, and watering must then be taken. But in winter-time a satifsactory development of the crop is only possible when artificial irradiation is applied. The usual Neon-light irradiators are most succesfull. In emergency cases, ordinary lamps can be used, but the light-intensity must then be very high. It is not necessary to give here more details about the technique of raising the crop and its irradiation in winter-time, since no special measures have to be taken and the general rules for hothouse culture can be followed.

Another very important detail, that must be mentioned in connection with this culture, is the danger of epidemic diseases in the spinach especially in winter-time when ventilation in the hothouse is

always lacking. All generally recommended measures were taken to prevent or suppress such epidemics, but it was not always entirely possible to suppress development of mildew or of *Botrytis*-blight.

Since in preliminary experiments it was observed that, in most soils, the natural amount of K and P was insufficient for an optimal development of the spinach or for an optimal mineralisation, it was considered safe to add these elements artificially at the moment of sowing the extraction-crop. An amount of 0.2 g K_2HPO_4 per kg of soil was always sufficient to abolish this deficiency.

As a rule it is not difficult to d e t e r m i n e w h e n t h e c r o p h a s t a k e n u p a l l a v a i l a b l e m i n e r a l i s e d n i t r o g e n. The rapid growth of the thickly sown crop ensures a rapid and complete exhaustion of the mineral nitrogen, and that moment is unmistakably marked by a yellow discoloration, cessation of growth and premature flowering of the spinach, all characteristic symptoms of N-deficiency. Therefore it does not require much training to be able to estimate the time of complete exhaustion rather accurately. The whole progress of the growth and the yellowing of the crop are as a rule so demonstrative, that it can often be used for a rather accurate prediction of the amount of mineralised nitrogen and of the intensity of mineralisation since exhaustion is reached sooner when the mineralisation is weak. In some exceptionally N-rich soils, as for instance some rich gardensoils and some marine deposite, exhaustion cannot be reached by one crop of spinach, and the sample must be sown twice for reasonable exhaustion. On the other hand, in very N-poor soils, complete exhaustion can be reached within 3 or 4 weeks, but in normal soils a vegetation-period of 5 to 6 weeks is required.

At the e n d o f t h e e x h a u s t i o n p e r i o d it is not sufficient to remove the crop by pulling it out. Too many fine roots would then remain in the soil and they should cause too much nitrogen uptake during their decay. A careful sieving of the whole soil with simultaneous removal of all visible rootlets is necessary. The easiest way to proceed is to shake off the soil gently from the roots of the plants above a sieve, holding them by their top ends and trying to prevent the breaking of the rootlets as much as possible.

Before the carefully sieved and mixed sample is replaced in the dish, small samples are taken from it for the determination of moisture and mineral nitrogen content at the start of the regeneration period since the exhaustion of the available nitrogen is never com-

plete. There seems to be a minimum limit of concentration in the soil, which the plants cannot remove even if the exhaustion period is prolonged. This minimum quantity varies in different soils. But when the exhaustion is performed properly, there is never more than 3 mg of water-soluble N per kg of dry soil left. Therefore this limit was adopted as a critical maximum allowed at the beginning of the regeneration.

The moisture content of the samples must be adjusted at this time to approximately 60 to 70% of the total moisture-holding capacity of the type of soil concerned.

For the regeneration-period the samples are brought in the same earthenware containers to a dark thermostatically controlled room, where a temperature of 29°C. is maintained and the atmosphere is approximately saturated with water. To save space, the dishes are put on racks extending from floor to ceiling, leaving only narrow interspace between the successive shelves. Because of this arrangement, it is strictly necessary to operate a small fan in the room, to attain regular distribution of temperature and moisture over the entire space. Maintenance of the very high humidity in this room is essential. Only in an atmosphere practically saturated with water, can evaporation from the soil be reduced to a negligible level, which renders any watering of the samples during the regeneration period superfluous. It must be strongly emphasized, that it is strictly necessary to reach so high a humidity since this is the only way to obtain a really undisturbed process of regeneration. It is useless to place open dishes filled with water in the room. Even a continuous dripping of water proved unsuccessful. The only effectual measure was the evaporation of water at a higher temperature than the average temperature in the room. For this purpose the following arrangement was designed and proved to operate satisfactorily: Heating of the room was performed by electric coils placed in the lower end of a wide pipe, fixed in a vertical position in the room, and open at both ends. At the top end of this pipe a small fan was fixed so as to maintain a continuous upward air flow through the pipe. Halfway up the pipe a thick piece of cloth was stretched in an inclined position and water from a perforated pipe-line was continuously dripping on the cloth, keeping it soaked, while warm air from the electric coil passed along it and caused a very rapid evaporation. By a proper regulation of the distance between the heater

and the cloth it was possible to adjust the evaporation accurately to ensure a nearly complete saturation of the atmosphere.

For the regularly repeated sampling of the soil during the regeneration period the same procedure was followed as at the start of the regeneration. The whole amount of soil in the container was mixed and sampled using the sectorial divider. The rest was then replaced in the container. Usually samples were taken once a week. For special studies sometimes shorter intervals were adopted.

These periodical samples had to be analysed for their moisture and mineralised nitrogen contents. About the first mentioned analysis no details have to be given since the ordinary methods were followed, but for the nitrogen analysis a complete description must be given because a method was applied in this investigation which deviated slightly from the standard prescriptions given in handbooks:

250 g of the soil (with the natural moisture content) are rotated for 2½ hours at room temperature on a shaker in a flask of 1000 ml volume, with 600 ml distilled water and 3 g of $CaSO_4$ (free from N) to aid a rapid and clear precipitation after the shaking. Then the soil was allowed to settle overnight. If insufficient clear solution was available above the precipitate, the whole flask had to be filtered. 300 ml of this clear solution was pipetted into a distillation flask of 1000 ml, 30 ml of a NaOH-solution of 30% by weight and 3 gr Devarda-alloy were added and the flask immediately connected to the cooler on the distillation-stand. The Devarda-alloy must be finely ground, to give a powder, that passes through a sieve with threads that are 0.17 mm apart. The composition of the Devarda-alloy is 59% Al, 39% Cu and 2% Zn. Before starting the actual distillation with heavy boiling, the flask must be heated very gently for 30 min. on a small flame of not more than 2 cm high. The flame may not be increased before the end of this period. The condensate is collected in an excess of 0.01 n H_2SO_4. The excess acid is titrated back by 0.01 n NaOH. The most succesful indicator used was that of Tashiro (4 parts 0.1% methylred and 1part 0.1% methyleneblue by volume, both as alcoholic solutions). Nearly as good was the mixture of 3 parts of 0.1% bromocresolgreen and 1 part of 0.2% methylred, also as solutions in 95% ethanol.

The determined contents of mineralised N can now be calculated as mg per l of water in the soil or per kg of dry soil. Both ways have advantages and disadvantages. In the first case the figures represent the concentration of N available to the plants, but this value fluctuates with the very variable amount of moisture in the soil. The second method of expression gives better comparable figures, equivalent to

the amount of available nitrogen per acre. But in soils with extreme moisture-holding capacity, as in the peaty soils, these figures may represent much lower concentrations than in ordinary soils, thus claiming better nitrogen-nutrition of the plants than really is offered in those soils.

IV. *The validity of the method and its value.* Although this method, which is based on the principle that provision of nitrogen to plants depends primarily on the course of the mineralisation of the humus and not on the amount of soluble nitrogen available at a certain moment in the soil, tries to follow this principle as strictly as possible, it cannot claim to be perfect. On the contrary, more than one shortcoming could be anticipated and still others were met when the experimental work proceeded. The most important defects will be discussed now.

A. Artificial conditions in the soil during regeneration. Conditions in the samples during exhaustion by the spinach as well as during regeneration of the mineralised nitrogen are not the natural conditions prevailing outdoors in the fields, but are considerably changed and entirely artificial. During the growth of the exhaustion crop the conditions had to be chosen to be ideal for a good and quick development of the crop. They are thus approximately the same as are maintained in all moderately heated hothouses of commercial growers. But during the regeneration period, not attempt was made to approximate natural conditions. On the contrary, the aim was to establish ideal conditions for an optimal mineralisation (crumbled, loose structure of the soil, optimal temperature and moisture content, very good aëration). Thus this method does not yield information about mineralisation as it is found in the soil in situ during the vegetation period, but about the potentially optimal mineralisation in the soil concerned. That is in some respects a severe shortcoming of the method, which it has in common with all methods using mixed samples for the determination of any metabolic activity of the soil. The information given by such methods cannot be directly applied to field conditions and must always be checked by field-trials or by statistical comparison with the results of practical farmers. But otherwise these methods give comparable results, which can scarcely be gained by the determination of the same property in the field, where discontinuity and local

variability of the soil interferes with the accuracy of the results. A reasonable accuracy of the field methods can therefore only be reached by increasing the number of multiple determinations to such a high extent, that the method becomes very laborious and expensive.

All methods using mixed samples, kept under artificial laboratory-conditions, are especially valuable for serial work but are much less valuable for fundamental research. And even for serial investigations the data obtained must be checked by comparations with the results of experimental plots or with special fieldmethods.

B. Influence of physical conditions of soil before sampling on the regeneration. While this method was designed as a laboratory method and all shortcomings of such methods have been accepted as inevitable, a zealous attempt was made to obtain really comparable and reproducible figures. However, in one respect this proved to be very difficult. Even the most carefull crumbling and mixing of the samples was insufficient to standardise the structure of the soil and the conditions for development of the microbes. The results obtained were always influenced by soil conditions in the field at the moment of sampling. This influence may last through the whole procedure of the determination. Such differences can be rather significant between samples taken under dry weather conditions in summer and those taken from the same plot in winter, when the soil is wet, uncovered by the crop and when the microbial activity is low. In Fig. 3 such a difference is shown for a heavy clay from a plot near Kampen, province of Overyssel. The two curves give the impression of belonging to two entirely different soils, but in reality only temporary changes in the conditions caused the marked difference.

The influence of the physical conditions of the soil may be demonstrated still more spectacularly by the curves reproduced in Figs. 4 and 5. Fig. 4 shows the regeneration-curves, both representing the same soil sample, crumbled and mixed as usual. One part of this sample was exhausted by spinach, the other part was percolated by water until approximately the same level of soluble nitrogen was left in it as in the first part; then it was partly dried and crumbled again. Then both parts were simultaneously placed in the regeneration thermostate. It is evident that the percolated part of the sample, that suffered very much in its structure and was badly pepti-

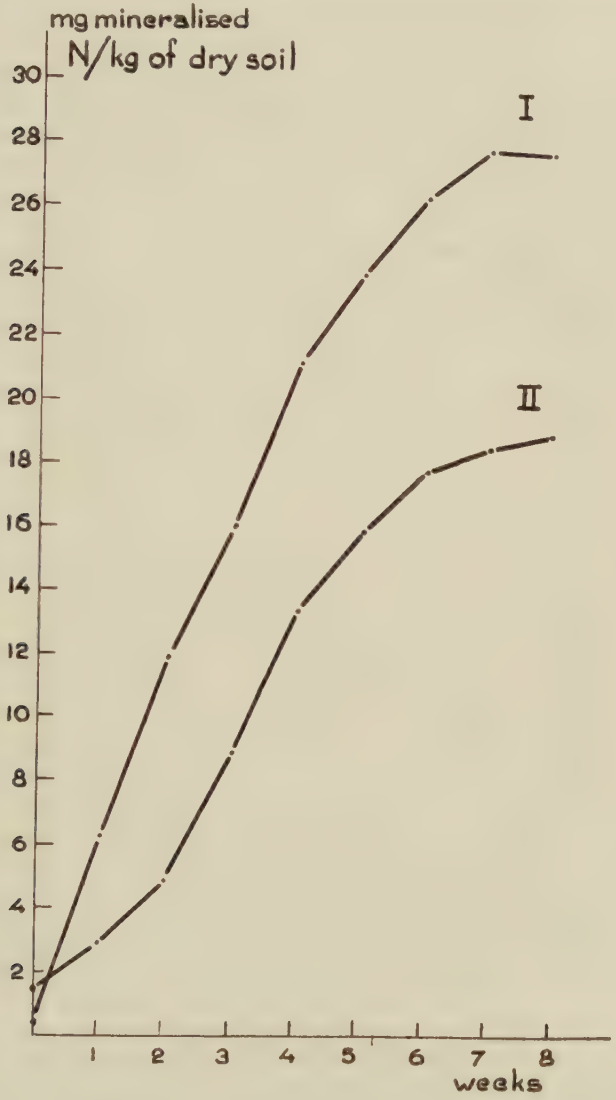

Fig. 3. Curves obtained from samples of the same plot, performing the determination under strictly comparable conditions.

I: Sample taken in August during fair weather. The soil was dry and had a crumbly structure (moisture content 17.2%).

II: Sample taken in December during a rainy period when the soil was practically water-logged (moisture content 34.7%).

sed during the treatment by water, had not overcome this damage during the entire period of regeneration. Macroscopically no much difference was visible between the two parts of the sample, but the internal structure of the soil crumbs remained different, consequently giving also very different rates of mineralisation. It proved to be easy to reverse this result by using the same two parts of the sample

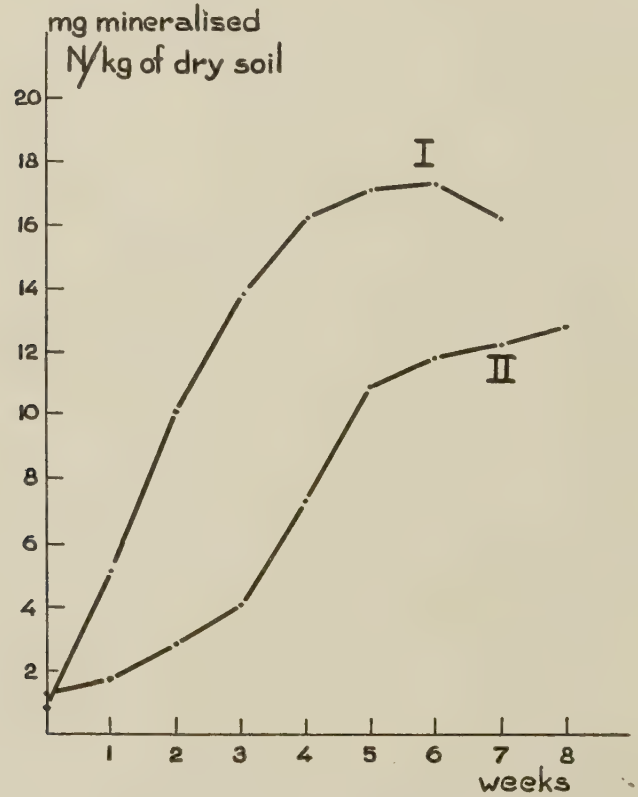

Fig. 4. Curves simultaneously obtained from one soil sample, devided in two parts.

Part I: Treated as usual by cultivating spinach on it.
Part II: Percolated by water.

for a second exhaustion and regeneration, but now using spinach for the part that first was leached by water, and percolation for the part that in the first experiment was sown to spinach. The curves obtained

are shown in Fig. 5. It is evident that the results of the regeneration are now exactly reversed.

Such marked differences as are demonstrated in both the abovementioned examples are found only in soils with a poor, week structure, but since the percentage of soils with a tendency to peptisation is rather high and smaller differences can be found in every soil, it was decided that samples should be taken only in periods of approximately equal conditions: all during the winter season (November–February), thus in wet and cold weather; or all in the period of the most rapid development of the microflora in the late spring (April

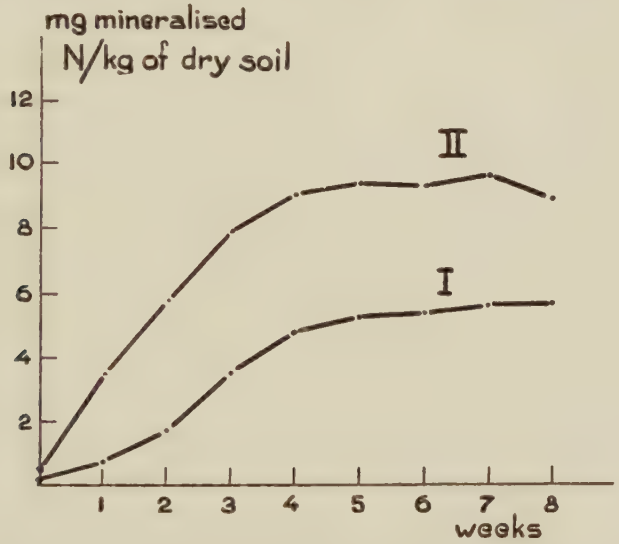

Fig. 5. Curves obtained in a second treatment of the same soil as show in Fig. 4, but with the exhaustion treatment reversed: thus using spinach on part II of the sample, previously leached out by water, and percolating part I, previously extracted by spinach.

and May) before the crops have exhausted the mineral nitrogen in most soils; or all during the second part of the summer when the soils are dry and nearly deprived of their mineralised nitrogen (July, August and September). However, the efficiency of the method is very much reduced hereby and the method is restricted to seasonal periods, which makes it much more expensive and requires a larger staff. For practical application it will presumably be necessary to extend the work over a larger part of the year, taking samples during

two of the three indicated periods or even in all three periods. But the results obtained in different periods must then be corrected to make them comparable. Such a correction will require many comparative analyses on the same soils, sampled in the different seasons. It will be necessary to establish such correction-factors empirically for each type of soil separately. The safest way will be not to use correction-factors found by other institutions for similar soils, but to determine them independently for each experimental station and for each regional investigation before starting the serial work. But it must be emphasized that samples should not be taken during the intermediate periods, i.e., in the late autumn (October) or in the early spring (March), or in June, when soil-conditions are changing rapidly and thus are very inconstant and variable. Also periods of very unusual extreme conditions (the first days after a heavy rainfall, severe droughts, periods of freezing weather and the first days after the thawing of frozen soil, etc.) must be avoided. Such short interruptions in the sampling, however, do not cause serious delays in the procedure of the work, on the contrary they may even be very welcome for overhauling the equipment and to make up arrears in the analytical work.

C. Only the first part of the regeneration-curve can be used for the determination of the nitrogen requirement of the soil. During the numerous determinations performed, using this method, it was always observed that only the first part of the regeneration gives smooth curves, approaching nearly straight lines, while later all curves bend over to the horizontal and become irregular with ups and downs. Only the first smooth and nearly straight part of the curves is therefore suitable for the estimation of the nitrogen requirement of the soil, since it is the slope of the curve in this period of regeneration that indicates the amount of available nitrogen that the plants can get from the soil, per unit of time, under conditions resembling those in the field, i.e. when no appreciable stock of soluble nitrogen is available. But unfortunately this part of the curve is often short, making the whole method less accurate and less reliable, since the slope of this part of the curve then depends on only 2 or 3 consecutive analyses. For some soils the straight part of the curve is longer, but nevertheless even these curves always start turning to the horizontal, simultaneously becoming less smooth, sometimes showing

very sharp ups and downs. The general trend of the curves to approach the horizontal is understandable, although this seeming decrease of the rate of mineralisation is in the nature of the case not really a decrease, since the amount of humus that can be readily mineralised cannot be reduced appreciably within such a short time and also the concentration of the products of metabolism is not nearly high enough to bring about a real decrease of the rate of fermentation. Buth the increase of the concentration of soluble nitrogen compounds gives other groups of microbes the opportunity to attack carbohydrates and other nitrogen-free organic compounds in the humus, synthesizing their own protoplasm. This multiplication of microbes at the expense of nitrogen-free organic compounds and of the mineralised nitrogen is the factor competing with the breakdown of the nitrogen containing compounds of the humus in the release of soluble nitrogen from the organic form. The higher the concentration of the soluble nitrogen becomes, the more microbial protoplasm is synthesized, retarding the rise in the soluble nitrogen-content. Consequently the curves, indicating this rise, turn more and more towards the horizontal during the regeneration.

Less understandable is the tendency of nearly all curves to show pronounced ups and downs in the later phase of the regeneration. These fluctuations indicate some irregular periodicity in the development of microbes and thus also in the uptake of mineralised nitrogen. They are not produced by fluctuations in any of the external growth factors but are spontaneously developed by the microbes, since they occur under strictly constant conditions of temperature, moisture, irradiation, etc. as well as in less accurately regulated cultures. Such spontaneously arising fluctuations are very common in the inorganic as well as in biological processes. Therefore it is not at all surprising that the mineralisation of the humus by microbes also shows very pronounced fluctuations. The ideal smooth curves shown in diagram in Fig. 1 are very rare; nearly always, some irregular ups and downs are observed in the later stages as shown in Figs. 3, 4 and 5. Yet these curves are examples of exceptional regularty. Irregularities in the later parts of the regeneration are usually more pronounced. Typical curves of the latter type are shown in Fig. 6.

Sometimes the ups and downs in the regeneration of nitrogen become perceptible unusually early, in the normally straight part of the curve, making a still greater part of the curve valueless for the

estimation of the nitrogen requirement, and thus reducing the accuracy of the method.

This very pronounced interchange of periods of predominating mineralisation with those of predominant microbial synthesis in the break-down of humus in soil, has already been reported by Gerret-

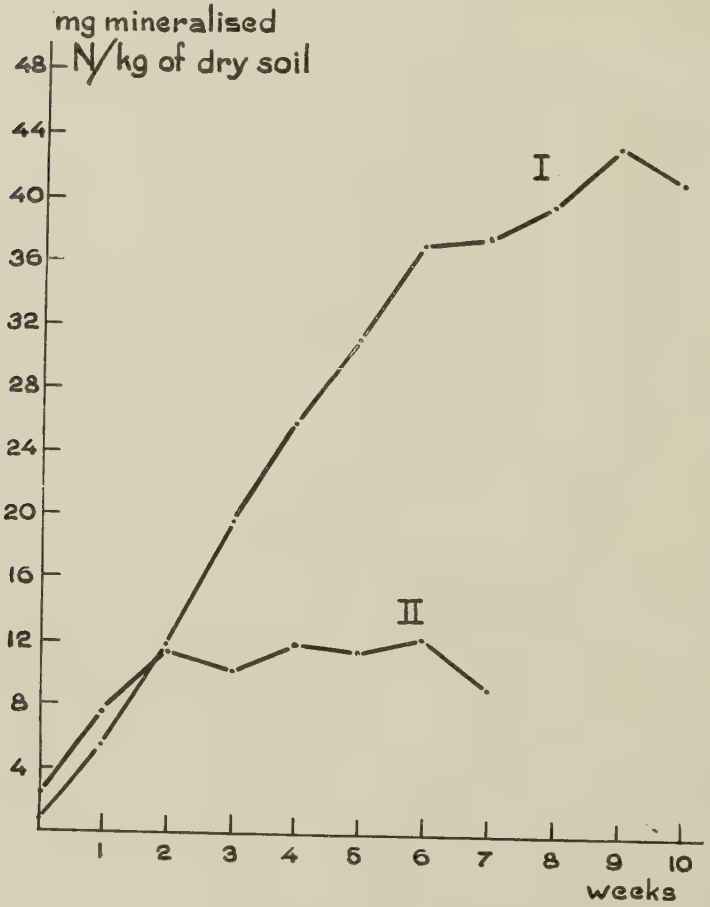

Fig. 6. Two curves of very common types, showing sharp ups and downs in later stages of the regeneration.

I: A steep long curve, characteristic of a very N-rich soil. The irregularities become evident only after 6 weeks of regeneration. A desirable type of curve.

II: A rather steep but short curve, characteristic of moderately N-rich soils. The irregularities disturb the curve after the second week of regeneration, making the determination very uncertain.

s e n [2]) for soil samples kept under similar conditions, without giving a complete explanation of the phenomenon.

D. Analytical determination of mineralised nitrogen is based not upon its availability to the plants, but upon its solubility in water. This is fundamentally a serious shortcoming of this method, as while it is true that all soluble nitrogen is available for the plants, the reverse is not valid. Part of the nitrogen — the NH_4-fraction — that is readily available to the plants is not entirely soluble in water, but is partly retained by the soil-particles as absorbed ions. But practically no troubles were caused hereby in ordinary types of soils in which the amount of ammonia is very low and thus very few ammonia-ions are available among the absorbed bases. Only in the especially ammonia-rich soils, mentioned when describing the method, does this fraction become important, and then it may not be neglected. In those cases, however, it proved to be possible to replace the ordinary water by a solution of *n* KCL or NaCl for the preparation of the soil-extracts for the Devarda-analyses. Hereby the absorbed NH_4-ions could almost entirely be replaced by the Na or K ions and the ammonia consequently was dissolved quantitatively. No troubles were met during the Devarda-analyses of these NaCl or KCl containing extracts.

E. The impossibility of removing the rootlets of the exhausting crop entirely from the soil-samples. At the time of sampling, sometimes the soil already contains many living rootlets, especially samples taken on grassland. But even more trouble is brought about by the rootlets of the spinach, since they are still living when the regeneration is started, while the roots of the original vegetation in the field are already partly decayed during the exhaustion period. Such undecayed fresh plant-tissue, that usually has a rather low nitrogen content, exerts the usual action of all N-poor organic material: in the first stages of its desintegration it absorbs much of the soluble nitrogen from the soil, reducing hereby the rate of release of the nitrogen from the humus. It is therefore quite understandable that the amount of fresh roots in the sample has a pronounced influence on the shape of the observed curves. For a correct operation of this method it is therefore essential to remove the small rootlets of the spinach completely before starting the regeneration. But, as already

mentioned, it proved to be impossible to do this completely. In very loose, sandy soils it was possible to remove nearly all rootlets if the sieving was done very accurately, but the heavier the soil is, the more of the very fine rootlets remain in the samples. Generally speaking, the heavier a soil is and the more fine roots were produced by the crop, the more is the mineralisation thus retarded. To date insufficient work has been done to establish quantitatively the necessary corrections for each type of soil, but this will have to be one of the most urgent measures for an improvement of the method, since now the heavy soils are always appreciably handicapped, compared with the sandy soils. But even when corrections for the main types of soils have been determined, they will always be only an approximation of the influence really exerted by the roots in each special case. Therefore these rootlets remaining in the samples constitute one of the most serious imperfections of the method, and no way to overcome it has yet been found.

F. The method is relatively laborious and expensive. This disadvantage of the method must be mentioned. It is true that it is much easier and cheaper to determine the nitrogen requirement of soils by this method, than by field trials and by collecting the experience of the farmers over many years, but compared with other analytical determinations in soil samples, the method outlined in this publication decidedly is one of the most laborious. The exhaustion of the soil by the spinach requires approximately 5 weeks, while the regeneration takes 6 to 7 weeks. The total duration of the determination therefore is at least 3 months Only when the mineral nitrogen content at the moment of sampling is very low, the especial exhaustion by spinach in cases of emergency may be left out, but for the sake of comparability it is never advisable to do that.

The costs of the total determination are especially raised by the necessity to have a hothouse with heating and irradiation arrangements and of a thermostatically regulated room with humidity control. But the required man-power is also very high, as each sample requires at least seven successive analyses. Until now insufficient serial work has been done with this method to be able to estimate the expenses per sample. The only experience, that could be gained was when the nitrogen requirements of the soils in the SW part of the Netherlands were studied in the years 1945–1947 after the recovery

of the land from flooding by sea-water during the war. But the experience collected there cannot give good information about the expenses, since the conditions for the work were very primitive, using an emergency laboratory, a very unsuitable hothouse, and a staff which was not yet sufficiently trained. In addition, most of the soils concerned were very difficult to handle, being exceptionally wet and sticky. Notwithstanding those very unfavourable conditions, nearly 200 samples could be analysed during one winter-season (from November to April) by laboratory personnel comprising one senior technical assistent (chief of the laboratory), two junior technical assistents and two laboratory helpers. It will undoubtedly be possible to increase the productivity considerably, using better equipment and trained personnel, and a still more important improvement will be reached if the macro-analyses can be replaced by micro-analyses, consequently reducing the work of sampling and transportation of the samples, the size of the hothouse and the incubator, and the amount of work at the laboratory.

V. *Discussion.* The conclusion seems to be justified that we now have a method that enables us to determine the rate of mineralisation of nitrogen in humus, with sufficient accuracy, much quicker and easier than was possible using only field-trials and the accumulated experience of farmers. It is true that the method described has more than one shortcoming, and only well-trained and critical investigators can get reliable results using the method; nevertheless it enables us to determine analytically the nitrogen requirement of soils. Until now only the requirements of K and P could be determined by laboratory methods.Thus this result is rather encouraging and satisfying, but the purposes for which this method can be used must also be considered, since it must be kept in mind that there is decidedly less need of analytical determination of the nitrogen requirement in soils, than that of K and P. The effect of nitrogen on the plants is much more visible than the effect of K or P. Therefore an experienced farmer can estimate the nitrogen he has to apply for a successfull development of the crops much more easily and accurately than the K and P fertilizers. In countries with active and sufficiently intelligent farmers, most of them will be able to estimate the requirement of nitrogen fairly well merely by experience. But in some special cases this laboratory-method can be very useful. Among

other things it will be a welcome air for investigations on the effect of organic manure and on the formation and disintegration of humus. This field of research is becoming more and more important and fundamental for agricultural science, where the main problem is no longer how to increase the yields, but how to maintain them on the present high level without too great an expense. For this research it is necessary to have a reliable method to determine the rate of mineralisation of nitrogenous compounds in humus.

A second application of this method is the investigation and evaluation of uncultivated land, or of land under very extensive and primitive exploitation, when measures must be taken to improve the methods of cultivation. In our country consequently only some tidal marshes, estuaries and shallow coastal waters will offer opportunities for such determinations before starting the enclosure and reclamation of those areas. But in vast and only partly cultivated countries all land for the expansion of the arable area must be selected on the score of a carefull investigation of the qualities of the soils concerned. Among these important qualities the nitrogen requirement occupies one of the most fundamental positions.

In addition to the two cases already mentioned, this method can become very valuable for the investigation of soils under special abnormal conditions, as for instance when soils by any catastrophe suddenly become spoiled, changing entirely the conditions in them. A very spectacular example of such a catastrophe is the flooding of old cultivated land by seawater as a result of inundations during the last war in the western Netherlands and in Flanders. Here this method was used for the first time on a moderately large scale, and it scored a success.

Summary

A new method is described for the determination of the rate of liberation of mineralised nitrogen from the organic form in humus. It is emphasized that only through this determination can a correct indication of the nitrogen-fertilisation requirements of the soils be obtained, since it is not the mineralised nitrogen available at a certain moment in the soil, that indicates the nitrogen supply for nutrition of plants but the amount of nitrogen that can be produced per unit of time by microbes from the large stock of nitrogen in the humus of the soil. Under natural conditions it is impossible to accumulate so much mineral nitrogen as to make this stock cover the total requirement of any crop during a whole season. Therefore the plants

depend for their nutrition during part of their growth on the continuous mineralisation of humus.

The method described is based on the consideration that the measurement of the rate of mineralisation is only reliable if it is performed during a period of very low concentration of mineral nitrogen in the soil. Therefore all mineral nitrogen must be extracted from the soil samples before the regeneration of the mineralised nitrogen can be measured. The extraction is done by cultivating a fast-growing crop on the samples and not by percolation with water, to prevent an unfavourable change in the structure of the soil. The regeneration is then measured by a series of analytical determinations of the water-soluble nitrogen in the samples, while these samples are kept in a room under controlled temperature and moisture, both adjusted to approximately the ideal conditions for the mineralisation.

The results of the consecutive analyses are plotted against time. The slope of the curve obtained is a measurement of the rate of liberation of nitrogen.

As far as could be contemplated theoretically and in the light of experience collected during the first serial application of this method, it is criticised and its value is discussed.

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THE BIOLOGICAL DETOXICATION OF 2: 4-DICHLOROPHENOXYACETIC ACID IN SOIL

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Introduction. The persistence in the soil of the new selective herbicide 2:4-dichlorophenoxyacetic acid (2:4-*D*) is one of its most important properties (Nutman, Thornton and Quastel) [9], but the ultimate recovery of the soil from its toxic action is of equal importance in practice. The velocity of this detoxication varies considerably with soil conditions, being accelerated by high soil moisture content, high temperatures and high organic content (Brown and Mitchell [3]), Kries [6]), Mitchell and Marth) [8]). There are also indications that high soil pH (excess lime) (Hanks [5]), Kries) [6]) greatly retards the detoxication. It has been suggested (Brown and Mitchell [3]), de Rose [4]), Hanks) [5]) that leaching, chemical or physical combination with soil constituents and biological decomposition may all be effective in normal soil detoxication. It would seem that leaching might play an important role under conditions of heavy rainfall and good drainage (Hanks) [5]), but cannot account for 2:4-*D* diaappearance in soil under storage (Brown and Mitchell) [3]). The part played by adsorption seems doubtful since here an immediate, not a slow delayed, action would be expected. This leaves chemical and biological decomposition as the two most probable alternatives. Studies on the persistence of 2:4-*D* in autoclaved soil (Brown and Mitchell) [3]) tend to support the latter.

Methods. In an attempt to obtain more direct evidence on this point, the soil perfusion technique of Lees and Quastel [7]) has been employed to study the kinetics of 2:4-*D* disappearance from

soil solution. In this technique, in which a solution of a known metabolite can be circulated in solution through normal soil for any required period of time, the fate of the metabolite can be followed with an accuracy dependent only on the analytical methods employed. It has been shown (Lees and Quastel) [7] that in biological processes in soils the time progress curve of the reaction has a characteristic form, showing a considerable initial lag followed by an exponential rise, both of which are characteristic of bacterial proliferation in pure culture. Such curves are regarded as strong evidence of a similar bacterial proliferation in the soil in response to the presence of the specific metabolite concerned. After these initial phases a steady maximum rate is reached at which point the soil is regarded as "saturated" with the particular bacteria responsible for the process concerned. In many cases this bacterially enriched soil retains its activity after drying and can be subsequently used to study the kinetics of the reaction by the Warburg manometric technique (Quastel) [11].

In the present preliminary investigations 200 ml of dilute solutions of 2:4-*D* (10–1,000 p.p.m.) have been perfused through garden loam (50 g of 1–4 mm fractions of the air-dried sieved soil) using a modified form of perfusion apparatus (Audus) [1] at room temperatures (20°C). Daily samples of the perfusate were taken during the perfusion. By using a suitable range of dilutions, reasonably accurate estimates of the 2:4-*D* content could be obtained by a biological assay depending on the growth inhibition of Cress (*Lepidium sativum*) roots studied by a technique already described (Audus and Quastel) [2].

Experimental Results. The results obtained appear in Fig. 1, where the growth inhibition produced by relevant dilutions of perfusate are plotted against the duration of the perfusion. With an initial concentration of 10 p.p.m. (Fig. 1*a*) there is a slight but significant decrease in toxicity over the first 3 to 4 days but from there onwards there is no significant change until after 10 days perfusion. Then a rapid disappearance of 2:4-*D* sets in, all toxicity being lost after another 4 days. In a subsequent perfusion through the same soil with the same amount and concentration of 2:4-*D* all toxicity was destroyed within two days. In a third perfusion with 200 ml of ten times the concentration (100 p.p.m.) all toxicity was lost in

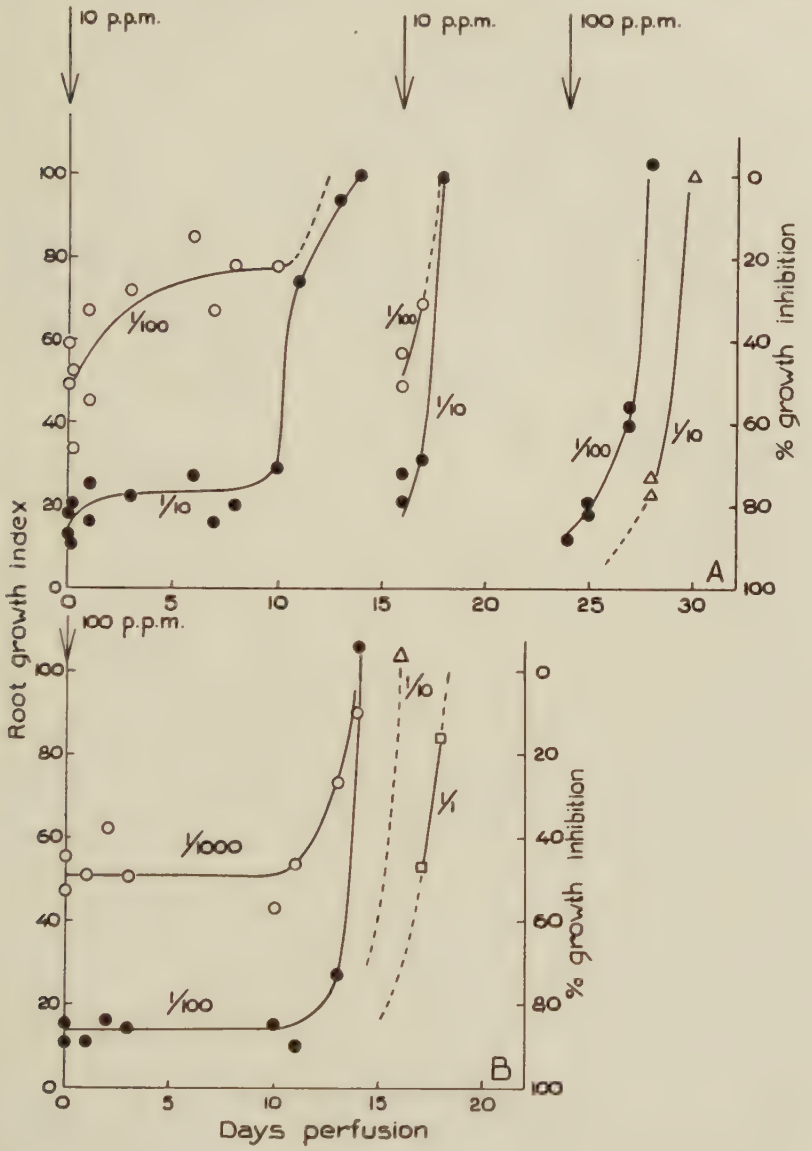

Fig. 1 (*a*) Curves showing the time course of toxicity of 2: 4-*D* solution on perfusion through garden loam. The fractions against the curves represent degrees of dilution of the assay solutions. The vertical arrows denote the time of introduction of fresh perfusate, the concentration of which is marked at the side. Initial perfusion with 10 p.p.m.

(*b*) As in (*a*) but with initial perfusion with 100 p.p.m.

5 days. Similar results were observed with an initial perfusion of 100 p.p.m. (Fig. 1*b*) where a lag of 11 to 12 days was obtained and complete detoxication within a further 6 days. Fig. 2 shows these results expressed as concentration-time curves. This long lag, followed by a rapid disappearance of the metabolite, is typical of the results of Quastel [10]) for biological processes in the soil and suggest that the detoxication of 2:4-*D* in soil is primarily biological. Proof of this should be obtained when the above experiments are repeated in the presence of a bacterial poison which should inhibit the detoxication process. This must await the adoption of a new

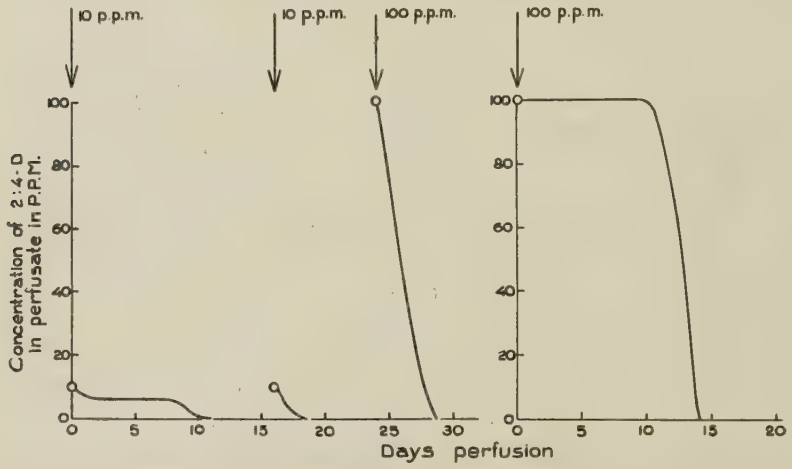

Fig. 2. The results of Fig. 1 smoothed and expressed as 2: 4-*D* concentration curves.

technique for the estimation of 2:4-*D*, since these poisons preclude, or at least greatly complicate, the use of biological assay methods.

The initial small decrease in toxicity of the dilute perfusate (10 p.p.m.) in the first few days of perfusion is probably due to adsorption onto soil colloids. A similar small adsorption followed by a typical biological decomposition has been shown to occur when coumarin is perfused through soil (Audus and Quastel) [2]).

After enriching the soil by perfusion with a concentration of 100 p.p.m., a solution of 1,000 p.p.m. was employed. The toxicity of this disappeared completely in 13 days. Assuming therefore a linear rate of decomposition by the organism concerned (Bacterial saturation of the soil) this corresponds to a maximum rate of approx-

imately 0.01 mg 2:4-*D* per g dry soil per hour. If this decomposition is due to an oxidative breakdown then it should be possible to detect or even measure the oxygen utilisation by the Warburg technique and thus determine the stoichiometric relationships between oxygen and 2:4-*D* in this breakdown. Using therefore a method devised by Quastel[11], this enriched soil was rapidly dried in a current of warm air and then placed, after sieving, in 5 gm aliquots in Warburg vessels. The oxygen uptakes of these samples were then followed at 35°C after moistening with 3 ml of 50, 100, 150 and 1,000 p.p.m. 2:4-*D*, using a similar soil sample moistened with 3 ml of distilled water as a control. All samples gave the same initial oxygen uptake of approximately 20 mm^3/g/hour, falling to half that value after 5 hours. These results suggest, although they are not offered as proof, that the detoxication of 2:4-*D* is not brought about by a process involving the absorption of oxygen but by one which may be hydrolytic in nature, It is of course possible that the activity of the organisms concerned is lost on drying and not restored on subsequent remoistening, but this is a subject for further research.

It is interesting that the detoxicated perfusate, when tested in the undiluted state, showed, in some cases, distinct evidence of a stimulation of root growth in Cress. With three perfusates (Original concentration of 2:4-*D* 10 p.p.m.) stimulations of 11, 26 and 57 per cent. were observed on a fresh weight basis, the roots being thinner but on the average twice the length of the controls. Similar observations have been made in soil (Kries)[6], Taylor)[12] and indicate that the products of 2:4-*D* breakdown may function as root growth stimulants. It is obvious that detailed microchemical studies on 2:4-*D* breakdown in these perfusion experiments is the next step to be undertaken.

Summary

By making use of a soil perfusion technique it has been shown that the detoxication of 2: 4-dichlorophenoxyacetic acid in garden loam is due almost entirely to the activity of microorganisms. Preliminary experiments suggest that the process does not involve oxidation. There are also indications that the decomposition products may include a root growth stimulant.

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EXPLORATORY POT EXPERIMENTS ON SENSITIVENESS OF DIFFERENT CROPS TO SODIUM *): A. SPINACH

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Introduction. It is a well-known fact, frequently discussed in the literature, that of the plant species sensitive to sodium, the reaction of the beet plant is by far the strongest. More recent investigations in America, Great Britain and also the Netherlands have given convincing evidence that the application of sodium to beet produces an effect, especially on weakly buffered soils, that may equal or even exceed the reaction to nitrogen and potassium. Consequently, sodium is being regarded more and more as an essential element for the nutrition of beet.

In respect of other crops the available data on the value of sodium are neither so extensive nor, on the whole, so positive. This fact stimulated us to investigate, in some orientating experiments, the sensitiveness to sodium of different crops, in order to obtain a clearer appreciation of the practical possibilities of sodium application.

It should be borne in mind that, for the estimation of the action of sodium, it is necessary to work with a standard which differs from that used for most other nutritional elements. As far as we know, there are no distinct symptoms of sodium deficiency in plants, unless certain symptoms, considered up to now as exclusively due to potassium deficiency, are wholly or partially identical with symptoms of sodium deficiency [3]), [4]). Sodium cannot be classified among the trace elements, because large quantities of sodium are necessary to produce a distinct effect and its

*) These experiments were commenced at the Laboratory for Agricultural Chemistry of the Agricultural College at Wageningen, in concert with Prof. Ir. J. Hudig, Director of the Institute. The experiments could not be finished there owing to interference of war conditions.

essentiality and physiological activity must, in the main, be measured by effects on yield and on the conditions of health and vigour of the crops [4]). Since the criteria for the action of sodium are not so clearly definable as those for other elements, it is particularly important to choose unambiguous experimental conditions, eliminating as completely as possible all factors that may influence or obscure the effect of sodium. We can see, in principle, three ways to investigate the action of sodium. The first is to make use of salt ssuch as sodium chloride, sodium sulphate or even sodium carbonate although these introduce, also, the accompanying anions. Hence if we aim to investigate the effect of sodium by itself we have to take this complication into account, because such anions cannot be deemed inactive. The presence of NaCl in the potash salts normally applied, gives practical significance to such experiments, and indeed common salt as such is applied, in England and in the U.S.A., amongst other places, on sites where the fertility of the soil makes nitrogen dressing superfluous.

Another method of investigation is to employ a cation-adsorbent such as dusarit, introduced by H u d i g and S c h u f f e l e n [10]). We adopted this method for sodium investigations in experiments with fodder- and sugar beets [7]), [8]) and H e l l i n g a also employed it [5]). The great advantage of this method is that it allows the testing of the effects of the cations almost individually, although one must use some nitrate and phosphate sources for the supply of nitrogen and phosphate.

Finally, sodium can be added with the nitrate employed for the nitrogen supply and its effect can be compared with another cation that is also fixed to nitrate, e.g. potassium or calcium. This can be done at a constant nitrate level and it prevents at the same time complications from other anions such as sulphate or chloride. The practical importance of these experiments is contained in the secondary reactions of the nitrate fertilizers due to the variation in nature of secondary ions [6]). As calcium is not only a constant component of all soil types, but is also supplied to the plant in the form of calcium nitrate — either directly or through conversion in the soil of other nitrogen compounds — the calcium-ion is, in our opinion, most appropriate for comparison with sodium in the nitrate nutrition of the plant in so far as fertilizer experiments are concerned. For physiological investigations it is the element potassium that requires special consideration in this respect.

On planning a few orientating pot experiments we decided upon a comparison of sodium nitrate and calcium nitrate in order to have at the same time a practical fertilizer test on the value of nitrate of soda. As, according to current conceptions, the effect of sodium is intimately connected with that of potassium, the comparison had also to be set up with different levels of potassium. In 1943 we carried out experiments with spinach, oats, barley, grass and flax which we hope to deal with successively. The scheme of the experi-

ments was kept on simple lines, but the investigation was made on dusarit-artificial soil with different levels of *K*-dusarit and addition of chemically pure nitrates on the one hand, and on a humus sandy soil to which potassium chloride was applied for the different *K*-levels and Chilean nitrate of soda and nitrate of lime for the nitrogen supply on the other.

Experimental section:

A. Spinach in Mitscherlich-pots on mixtures of dusarit-glass sand.

Pots with 5.8 kg glass sand + 300 g dusarit; water supply at 60% of the water holding capacity.

Basic dressing: 1.4 g $CaHPO_4.2\,H_2O$, 45 mg $FeCl_3$ 150 mg $MnSO_4.4\,H_2O$, 150 mg $CuSO_4.5\,H_2O$, 15 mg borax.

Potassium: levels of 1, 3 and 8% K-dusarit; the mixture contained furthermore 5% Mg-dusarit and was made up to 100% with Ca-dusarit. The pH of this mixture was 6.2.

Nitrogen: 1200 mg N in the form of $NaNO_3$ and $Ca(NO_3)_2$, as follows: 400 mg at the beginning of the experiment and the remainder in two portions as top-dressing.

Pots in triplicate.

The pots were sown twice as follows: — sharp seeds (broad leaf) in the spring, the production of which was gathered in one month's time, and round seeds in the summer.

The spring spinach developed very thriftily. The plants with sodium nitrate were soon outstanding in regard to abundant foliage and lighter greenish leaf colour, while the plants with calcium nitrate were characterized by small, compact and dark-greenish leaves, and in some instances by spotty appearance. Only on calcium nitrate with 8% K-dusarit did the plants of this series develop more abundant and lighter greenish leaves. Table I shows that the yields of the $NaNO_3$ plants were appreciably higher than those of $Ca(NO_3)_2$ plants and furthermore that the influence of potassium was only weak in presence of sodium nitrate, but much more distinct in the presence of calcium nitrate. Nevertheless calcium nitrate, even at the highest K-level, did not produce results comparable with nitrate of soda at the lowest K-level (Fig. 1).

No fresh potassium was applied to the summer spinach of the dusarit pots, so that the plants had to depend on the old stock; in

addition 800 mg N was added per pot. Owing to a persistent incidence of black leg, the plants of several pots grew defectively, making the replications more irregular. In the yields shown in table I these pots have been left out of consideration, so that the figures represent, as a rule, the averages of two pots only. Nevertheless this series presented essentially the same picture as the spring spinach but with a slightly more pronounced effect of the highest K-levels which can be explained by exhaustion of the potassium by the preceding crop from the pots receiving the lower K-levels (Fig. 2).

TABLE I

Yields of spinach on dusarit in g per pot

	fresh weight		dry weight	
	$NaNO_3$	$Ca(NO_3)_2$	$NaNO_3$	$Ca(NO_3)_2$
Spring spinach				
1% K	222 ± 6	85 ± 3	12.9 ± 0.4	7.7 ± 0.2
3% K	236 ± 6	126 ± 2	13.8 ± 0.2	9.2 ± 0.3
8% K	253 ± 4	160 ± 11	14.0 ± 0.3	10.5 ± 0.3
Summer spinach				
1% K	127 ± 1	37 ± 1	10.3 ± 0.4	5.8 ± 0.2
3% K	137 ± 2	52 ± 4	12.7 ± 0.5	7.2 ± 0.5
8% K	156 ± 6	124 ± 9	14.2 ± 0.5	12.4 ± 0.8

B. Spinach in Mitscherlich pots on humus sandy soil.

We employed soil originating from some zero plots of a potash trial field at Ruurlo (NGe 73), acquired by courtesy of Ir. O. J. Cleveringa. As the same soil was also employed in pot experiments with other crops, the analytic data are recorded below:

Loss on ignition 8.7%

pH 5.0

P-figure 6.5 (water-soluble phosphate; mg P_2O_5 per 100 g).

Per 100 g of dry soil the following quantities were estimated by extraction with sodium acetate solution of pH 4.8:

8 mg K, 9.7 mg Na, 103 mg Ca, 3.4 mg Mg.,

4.8 mg nitrate and 0.6 mg NH_4.

Water holding capacity about 30%.

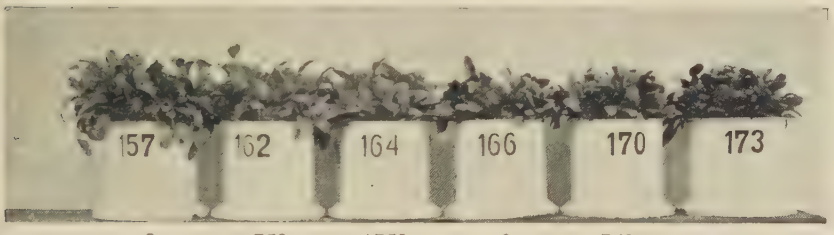
Pots with 4.5 kg of dry soil, kept at 60% of the water holding capacity. Execution in triplicate.

1% K 3% K 8% K 1% K 3% K 8% K
sodium nitrate calcium nitrate

Fig. 1 Spring spinach on dusarit

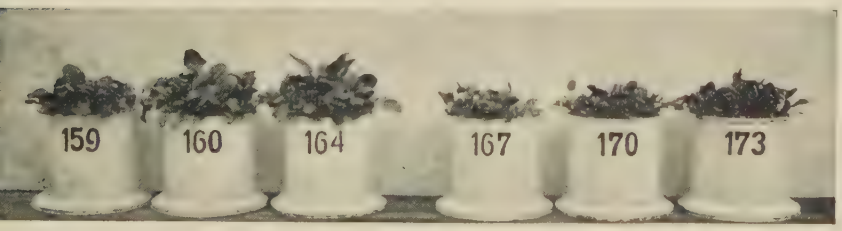
1% K 3% K 8% K 1% K 3% K 8% K
sodium nitrate calcium nitrate

Fig. 2 Summer spinach on dusarit



0 750 1750 0 750 1750 mg KCl
Chilean nitrate nitrate of lime

Fig. 3 Spring spinach on soil



0 750 1750 0 750 1750 mg KCl
Chilean nitrate nitrate of lime

Fig. 4 Summer spinach on soil

tegenover blz. 40

Basic dressing: 1.4 g $CaHPO_4.2\,H_2O$, 750 mg $MgSO_4.7\,H_2O$, 150 mg $MnSO_4.4\,H_2O$, 150 mg $CuSO_4.5\,H_2O$, 15 mg borax.

Potassium: levels of 0, 750 and 1750 mg KCl of which 350 and 750 mg respectively as top-dressing.

Nitrogen: 1200 mg N in the form of Chilean nitrate of soda and nitrate of lime respectively, as follows: — 400 mg at the beginning of the experiment and the remainder in two portions as top-dressing.

These pots were sown in the same way as those of series A. The initial development of the spring spinach was less satisfactory than that on glass-sand dusarit. This was probably due to the defective water economy of this sandy soil (in hot weather the upper soil layers dried out; the lower layers failed to supply sufficient water and consequently difficulties arose in the germination stage). This also caused delay of growth at first, but after a few weeks the plants recovered and the same picture as in the A-series developed. The plants supplied with Chilean nitrate were distinctly ahead in development and presented a sounder appearance and a brighter greenish colour than the plants supplied with nitrate of lime (Fig. 3).

In the early weeks potassium chloride reduced the growth of all pots to an appreciable extent, but with nitrate of lime it finally gave an improvement of state and yield. The Chilean nitrate pots without KCl remained the best throughout the whole growth period and this is quite evident from the yield figures (Table II).

TABLE II

Yields of spinach on humus sandy soil in g per pot				
	fresh weight		dry weight	
	Chil. Nitr.	Nitr. of lime	Chil. Nitr.	Nitr. of lime
No K	119 ± 16	68 ± 4	8.6 ± 1.0	5.9 ± 0.3
750 mg KCl	109 ± 15	81 ± 1	7.6 ± 0.8	6.6 ± 0.1
1750 „ „	95 ± 6	89 ± 1	6.8 ± 0.3	7.2 ± 0.1

On account of the bad germination and early development of the summer spinach due to black leg, which developed in spite of renewed soil working and second seed application, these pots received no further addition of either water or fertilizers. We must confine ourselves therefore to a photographic picture of these pots which inspite of all shows a pronounced sodium effect (Fig. 4).

Analyses: Table III and Fig. 5 and 6 show the contents of Na, K and Ca in dry matter of both series *).

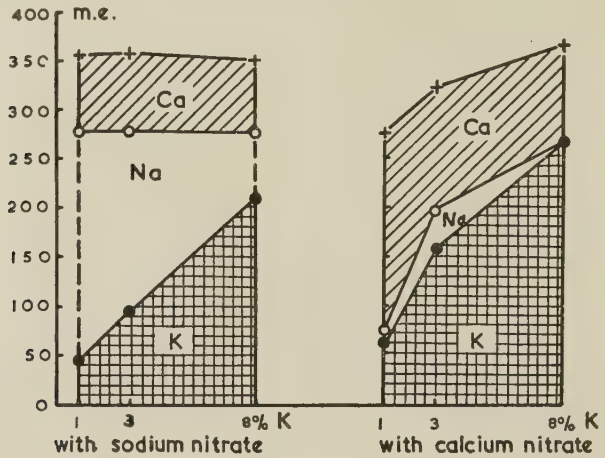


Fig. 5. Composition of spring spinach on dusarit-glass-sand (in m.e. per 100 g of dry matter).

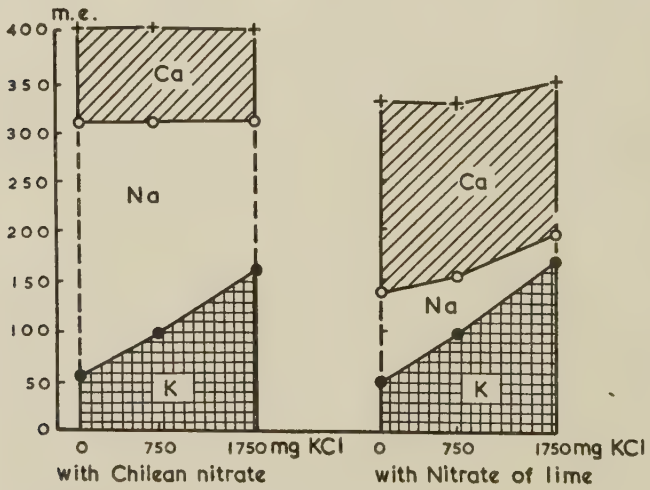


Fig. 6. Composition of spring spinach on soil (in m.e. per 100 g of dry matter).

There is every evidence that the mineral composition of spinach is subject to heavy fluctuations and depends largely on the supply of

*) Na and K were determined in flame-photometer (Perkins and Elmer); Ca as oxalate.

the corresponding cations in the nutrition. We have previously established the same fact in fodder beet [7]) and sugar beet [8]). On comparing series A. and B. we find little influence of the medium of growth on the uptake of ions, except that the plants on sandy soil, grown with nitrate of lime, had a higher sodium content owing to the relatively high natural Na content of the soil employed. The differences between the spring and summer spinach may be explained in part as due to variety, but they are no doubt determined in the main by different conditions of nutritional level and temperature. As no fresh potassium was added to series A, the available stock was already partially depleted by the spring crop (declining K contents in summer spinach); in the contents of Na and Ca we find, on the contrary, a rise owing to accumulation of these elements from the nitrate dressing.

In all groups with calcium nitrate the content of univalent ions depends closely upon the supply of K ions, but there is an almost constant total amount of sodium and potassium in the groups with sodium nitrate; this might point to saturation of the plant with univalent ions.

TABLE III

Composition of spinach (in m.e. per 100 g dry matter)						
	Spring spinach			Summer spinach		
	Na	K	Ca	Na	K	Ca
A. Series on dusarit						
$NaNO_3$ 1% K	232	43	81	249	14	58
3% K	183	94	78	230	21	56
8% K	63	206	77	189	52	46
$Ca(NO_3)_2$ 1% K	10	63	200	9	22	273
3% K	38	158	126	9	31	275
8% K	2	258	104	8	88	217
B. Soil series						
Chil. Nitr. no K	256	54	93	221	67	79
750 mg KCl	217	94	95	129	152	74
1750 „ „	150	159	93	98	190	75
Nitr. of Lime no K	89	50	192	59	35	217
750 mg KCl	58	95	173	26	112	194
1750 „ „	31	170	148	14	168	165

As a comparison with the above analyses which all refer to plant material from our pot experiments, we also investigated a few

samples of spinach delivered by a greengrocer, so as to give an idea of the normal market-garden products:

1. April 1948: 3 m.e. Na, 280 m.e. K and 55 m.e. Ca
2. 15th Oct. 1948: 40 m.e. Na, 157 m.e. K and 66 m.e. Ca
3. 19th Oct. 1948: 38 m.e. Na, 149 m.e. K and 60 m.e. Ca

all in m.e. per 100 g dry matter.

Samples 2 and 3 have probably one and the same origin, as they were purchased in the same week and show an almost identical composition. A striking point is that in these samples potassium is in the largest proportion by far, especially in sample 1. It is not the place here to couple certain conclusions to the above, but the fact in itself may be commended to the attention of nutrition specialists for further investigation.

Discussion: Spinach (*Spin. Oleracea*) belongs to the family of the *Chenopodiaceae* and has a preference for univalent ions (K and Na) in common with the other members of this family. E. Wolff [11]); (p. 101) published even in 1871 some ash-analyses showing that this plant had a well developed ability to absorb sodium; for instance, in one sample (no. 51) 31.42% Na_2O against 23,43% K_2O, and in another (no. 52) 39.16% Na_2O against 9.69% K_2O. Recent analyses by Davidson and Le Clerc [2]) show for six spinach samples contents of 4.14 — 7.75% K and 0.7 — 2.37% Na (on dry weight).

Wallace, Toth and Bear [12]) count spinach among the "plants that accumulate Na, even if the nutrient substrate is almost free of Na". Also in our pot experiments spinach showed a strong ability to take up sodium with an almost equivalent replacement between sodium and potassium. It is true, however, that the plants have a certain preference for potassium, on account of which the Na content of the plants is reduced rather strongly in the presence of increasing K supply. The same preference for potassium was observed by Collander [1]) who found that spinach plants in water cultures that were supplied with equivalent amounts of potassium and sodium, assimilated these elements in a ratio of 5 or more to 1. Collander determined furthermore in all *Chenopodiaceae* a remarkably high uptake of magnesium, but a slight uptake of calcium. Our experiments show, however, that the uptake of calcium is only low in the presence of an adequately large supply of univalent ions.

Otherwise the meagre literature on the effect of sodium on the yield of spinach is not always consistent. Schuphan [9]) ascertained both with NaCl and Na_2SO_4 a favourable development of spinach and improvement in quality, but Harmer and Benne [4]), who carried out extensive experiments with NaCl dressings to muck soils, state that "spinach showed no benefit from the use of salt" and even classified spinach in the group of crops that "have none to slight benefit from sodium even in deficiency of potash".

Our pot experiments showed a strong yield effect in spinach dressed with sodium and there can be no doubt of the existence of a physiological function of sodium in this plant species. With respect to the contents of sodium and potassium in the plants, it is apparent that, if the total sum of these elements is left at a constant level, sodium gives yield effects which almost equal those of potassium. As an example the following yields of spring spinach, covering plants with total contents of Na + K of about 270 m.e. (per 100 g dry matter), may be quoted:

Group	Contents K/Na	yield of dry matter
1. $NaNO_3$ — 1% K	0.2	12.9 g
2. $NaNO_3$ — 3% K	0.5	13.8 g
3. $NaNO_3$ — 8% K	3.3	14.0 g
6. $Ca(NO_3)_2$ — 8% K	very high	10.5 g

Only in the event of strong deficit in K-content the yields due to $NaNO_3$-nutrition begin to lag behind (see also summer spinach — K/Na below 0.1); this indicates therefore K-deficiency. On the other hand, the yield in group 6 (calcium nitrate with 8% K) remains appreciably below the maximum and this is accompanied by an extremely low sodium content. The latter case might be interpreted as sodium-deficiency in the nutrition of spinach.

With regard to calcium it may be concluded that a shift in the plant composition from univalent ions to the bivalent calcium is coupled with a more compact growth and a correspondingly higher content of dry matter. As, however, the total yield of dry matter per pot is by far best in the plants with a low content of dry matter, i.e. those having an ample supply of K and/or Na, high contents of dry matter must be interpreted, in our opinion, as a sign of

retarded development. Except in one single case it was found that a high content of Ca is invariably coupled with low yields.

As regards fertilizer effect sodium and potassium cannot be assumed to be equivalent without some reserve, because it is apparent that from a mixture of both elements the plant assimilates potassium proportionately more readily than sodium. In the dusarit series the K-levels were: 9.5, 19.5 and 33 m.e. K per pot respectively, while in the soil series the K-levels were: 5.3, 16.0 and 42.7 m.e. K per pot. Against this the Na-dose carried with the nitrate of soda amounted to 85.7 m.e. The intake of potassium, computed as that proportion of the fertilizer-K which is taken up by the plant, is consequently higher than that of sodium. However, when it is possible to apply sodium on a liberal scale, as was done here as a secondary component of the nitrate fertilizer, the intake of sodium can be much increased and it then meets a quite considerable part of the need of univalent ions, so that only a moderate dressing of potassium is needed. Even if there is adequate potassium available, it appears that an ample supply of sodium can increase the yield further, so that from an economic point of view sodium has a dual importance. Moreover, sodium may be assumed to cause a certain improvement in quality.

It is not yet clear to us what role is played by the chloride in the nutrition of spinach. Schuphan [9]) takes this crop to belong to the chlorophillic plants, but weakens this conclusion by the statement that the reaction to chloride or sulphate strongly depends on the climate (rainfall) *) Harmer and Benne [4]) obtained in their experiments no beneficial effect from common salt, while in our pot experiments with sandy soil potassium chloride had a distinctly depressive effect. Combined with sodium nitrate-nutrition this effect remained preserved in the yields, while the plants with calcium nitrate got over it at a late stage. For this reason the possibility should be taken into account that the negative results of NaCl on spinach, obtained by Harmer and Benne, do not point so much to indifference towards sodium, but rather to sen-

*) It should be noted that Schuphan's scheme of experiments and his conclusions are subject to criticism. We would point out that in his "Grossbeerener Versuch" the high dose of potassium gave a distinct yield depression in case of bad water supply which contradicts Schuphan's general conclusion that chloride fertilizers have a relatively better reaction in dry seasons than in wet seasons.

sitiveness to chloride. An observation that may be related in this connection is that dusarit-glass sand mixtures are an ideal growth medium for this crop; this must be attributed in the first place to the good water supply of such mixtures. The humus sandy soil, of a very common type in Holland, manifested, however, a bad water economy and it was on this soil that salt addition (nitrate + potassium chloride) had an impairing effect.

Summary

In orientating pot trials, carried out partly on soil and partly on an artificial soil-mixture, spinach showed a pronounced ability to take up sodium when dressed with sodium nitrate. The crop benefited much by this type of fertilizer both in yield and outward appearance, as compared with controls receiving calcium nitrate.

In spinach sodium can replace potassium to a large extent, but it appears that, when receiving an equal supply of sodium and potassium, this plant prefers potassium. The use of sodium, supplied as a secondary constituent of nitrate fertilizers, is found to have economic importance, because it reduces the plant's need of potassium and improves the yields, even in presence of adequate potassium.

In pot experiments with soil the dressing of potassium chloride in combination with Chilean nitrate gave negative results. Chilean nitrate alone, however, produced the highest yields of the series. Combined with nitrate of lime, potassium chloride initially depressed the growth, but showed in the end a slight increase of yield. As the water supplying power of this soil was more or less deficient, it is deemed possible that the negative effect of potassium chloride is due to the action of salt-concentration.

It is pointed out that the normally grown spinach from the market-gardening centres destined for consumption may contain very high contents of potassium.

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STUDIES WITH SWEET CORN

I. COLD TREATMENT AND GERMINATION

by GORDON HASKELL

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When a crop like sweet corn is grown in a cold climate, the germination of the seeds are often poor, especially if they are sown in the early months of the year. It is therefore necessary to improve germination in colder conditions and thus produce hardier cold-resistant strains.

Haskell and Singleton (1949) have already shown that there is a correlation between the germination of early field sowings and of those subjected to cold treatment. The following investigation was therefore carried out with a number of inbred lines of sweet corn to determine the factors influencing germination at low temperatures.

Materials and methods. Five inbred lines of sweet corn were used. All carried the recessive sugary gene *su* for endosperm constitution. These were C 4, C 40, T 51 and C 95. A double cross dent hybrid, Conn 520, carrying the dominant gene for starchy endosperm, *Su*, was also used. The seeds were sown in seed boxes containing soil from a field recently under sweet and dent corn. Fifty seeds of each line were used for each test. Seeds of each line were sown paired rows and lightly covered with soil. The lines were kept in the same order in each seedbox to reduce competition effects should there be any. Their position was according to their maturity, viz.: –

Early: C 4, C 40, P 51, Conn. 520, C 95: Late.

Thirteen seed-boxes were prepared in this manner. One box was kept as control in a greenhouse with a temperature of approximately 75°F (84°C). Under this condition germination is approximately one hundred per cent. Six boxes were kept at a temperature of 50°F

(10°C) and six at a temperature of 40°F (5°C). The boxes were watered daily or whenever necessary, to keep the soil reasonably damp. This was sufficient to permit normal emergence. It has been found (Haskell 1949) that moisture content of the seeds is not a primary factor in effecting cold hardiness.

After two days one seed-box from each of the cold temperatures was transferred to the greenhouse. Subsequently boxes were removed from both temperatures at 4, 8, 16, 24 and 32 days from commencement of treatment.

To determine germination under more sterile conditions, the experiment was repeated using sand instead of soil as a medium more free from soil organisms. Sand also differs from soil in its mechanical properties especially in relation to water evaporation and drainage.

Germination was the emergence measured by the maximum number of seeds producing plumules above soil or sand level. Table I gives the percentage germinations after various lengths of treatment in the two temperatures. The germinations of four lines are given in Fig. 1(*a–d*). Fig. 2 shows the mean germinations of five inbreds after different treatments.

TABLE I

Percentage germination of corn subjected to cold													
	Pedigree	Con-trol.	Days in 50°F Room						Days in 40°F Room					
			2	4	8	16	24	32	2	4	8	16	24	32
Soil	C 4	100	100	98	98	86	32	22	96	100	96	98	60	24
	C 40	86	88	74	38	4	0	0	98	74	56	26	6	0
	P 51	82	82	56	34	4	0	0	88	64	58	18	2	0
	T 51	84	64	56	28	2	0	0	68	54	38	10	0	0
	C 95	72	70	58	18	6	0	0	70	70	42	20	0	0
	Mean	**84.8**	**80.8**	**68.2**	**43.2**	**20.4**	**6.4**	**4.4**	**88.0**	**72.4**	**58.0**	**34.4**	**13.6**	**4.8**
	Conn. 520	82	84	56	8	0	0	0	94	78	46	10	0	0
Sand	C 4	98	100	98	100	92	84	84	98	100	98	88	62	44
	C 40	100	88	86	76	50	42	52	66	30	18	8	8	2
	P 51	90	92	68	80	52	44	22	66	40	28	12	0	0
	T 51	80	74	52	58	46	44	20	48	44	22	20	6	2
	C 95	82	60	20	54	32	30	20	56	38	22	2	2	0
	Mean	**90.0**	**82.8**	**64.8**	**73.6**	**52.4**	**48.8**	**39.6**	**66.8**	**56.4**	**37.6**	**26.0**	**15.6**	**8.8**
	Conn. 520	96	90	76	86	84	50	60†	72†	88†	64†	44†	16†	2

†) based on 20 seeds only.

Germination in soil. The behaviour of the lines in soil for both 50°F and 40°F treatments is reasonably parallel. In general, the longer

seeds remain in the cold the smaller is the number that germinate after removal to warmer conditions. This is due to the seeds dying in the cold while germinating slowly below soil.

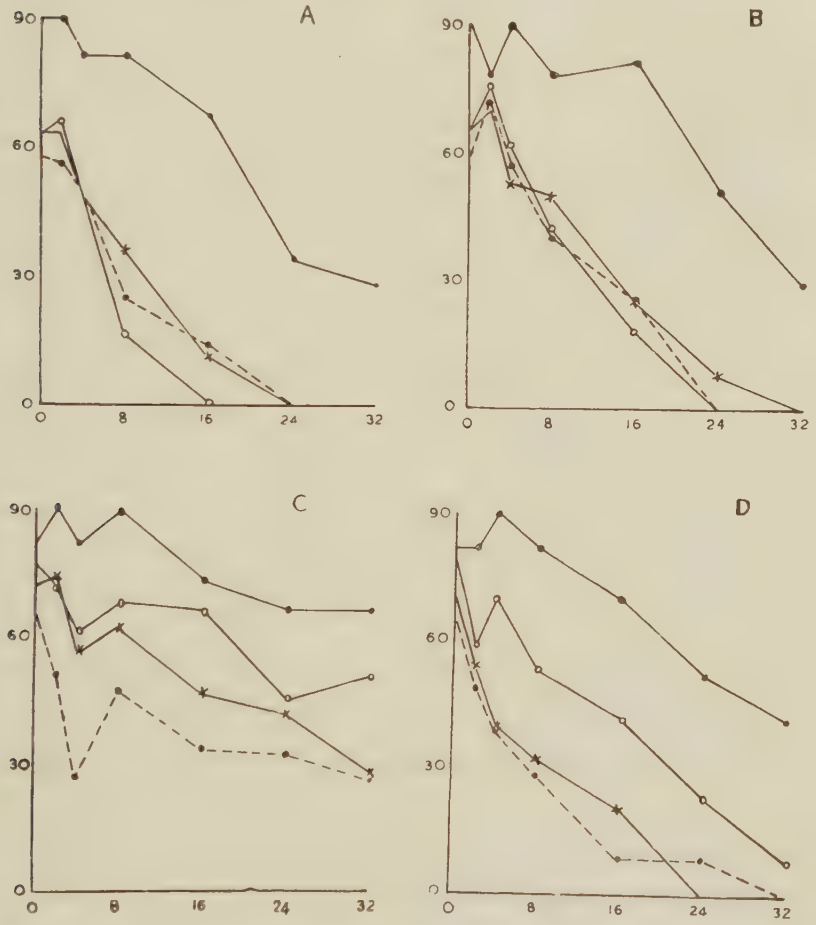

Fig. 1. The germination of three inbred sweet corn strains and a hybrid dent corn after subjection to various tengths of time in cold rooms.

(*A*) 50° room using soil in the seedboxes.
(*B*) 40° ,, ,, soil ,, ,, ,,
(*C*) 50° ,, ,, sand ,, ,, ,,
(*D*) 40° ,, ,, sand ,, ,, ,,

Abscissae: Germination expressed in angles;
Co-ordinates: Days in cold room.

—●— C 4 —×— P 51
—○— Conn 520 —●— C 95

There are, however, differences in rate of killing according to the genetical constitution of the lines. For instance, C 4 has a slower rate of killing off than the other inbreds or the dent hybrid. Its initial germination is somewhat higher than that of the others and this may account for the better behaviour of C 4 throughout the gamut

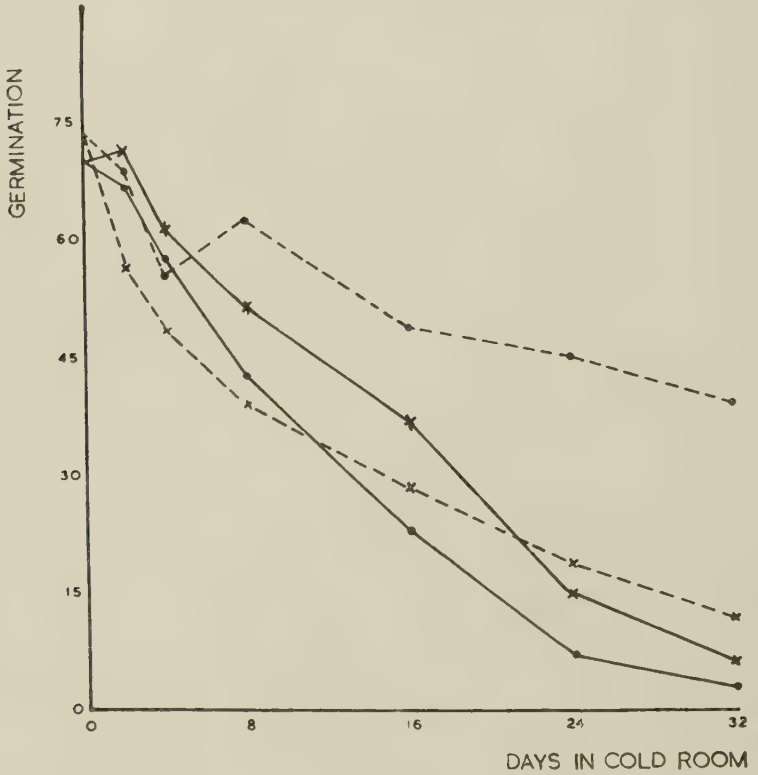

Fig. 2. Mean germination of five inbred strains of sweet corn. Subjected to cold room treatment.
Germination is expressed in angles.
——●—— 50° in soil — — — ● — — —50° in sand
——×—— 40° in soil — — — × — — —40° in sand

of treatments, although it is already known to be a very early and hardy inbred.

At both 50°F and 40°F all lines except C 4 react rather similarly. At both temperatures the starchy hybrid Conn. 520 failed to germinate after 24 or more days in the cold rooms. Thus genetical

factors for cold resistance are more important than whether the seed is hybrid or inbred, or the endosperm is starchy (*Su/Su/Su*) or sugary (*su su su*). T 51 is an extracted inbred from P 51 and slightly earlier. It reacts like P 51 but has slightly poorer germination throughout.

The means of the five inbreds for both 50°F and 40°F run fairly parallel to one another but the germinations at the lower temperature are consistantly greater. One form of so-called "cold-hardiness" in maize is thus *not only ability to withstand low temperatures at germination but also ability to withstand the activities of pathogenic soil organisms at these low temperatures*. Increased microbiological antagonism to these pathogens masks the real effect of the higher temperature on germination (H e n r y, 1932).

Germination in sand. In the sand experiments the graphs (Figures 1, *c* and *d*) for 50° and 40° are unlike each other. At 50°F only a slow decrease in germination occurs, while at 40°F the slopes are much steeper and, on the whole, not unlike those from both the 40° and 50° soil treatments.

As before, C 4 stands out in its resistance. In soil Conn 520 had been easily susceptible and no better than the inbred sweet lines but in the sand test it did somewhat better than the inbreds, other than C 4.

Analysis of results. To determine the relative importance of the various factors an analysis of variance was made on the original data converted to angles (M a t h e r 1947). The analysis is given in Table II.

All the factors analysed and all their first and second order interactions are highly significant. The single third-order interaction of length of time in cold room — temperature of cold room — medium used and varietal differences (i.e. the error item) is not.

The mean squares show that length of treatment has greatest effect, and interaction of temperature with germinating medium is also important even more than the individual effect of temperature and media. This agrees with the known reaction of soil pathogens in different media at different temperatures. Column 5 in Table II indicates the importance of the various experimental factors.

The regression of germination for each type of treatment on

TABLE II

Analysis of Variance of Data in Table I Transformed to Angles					
	S.S.	*N*	X^2	*P*	*M.S.*
Length of treatment *L*	49427.51	5	3011.30	*H.S.*	9885.50
Temperature . . . . *T*	1279.25	1	77.93	*H.S.*	1279.25
Medium . . . . . *M*	3143.47	1	191.51	*H.S.*	3143.47
Varieties . . . . . *V*	27742.13	5	1690.15	*H.S.*	5548.42
L — *T*	880.29	5	53.63	*H.S.*	176.05
L — *M*	4765.53	5	290.33	*H.S.*	953.10
L — *V*	1884.47	25	114.80	*H.S.*	75.37
T — *M*	6285.84	1	382.95	*H.S.*	6285.84
T — *V*	366.02	5	22.30	.001	73.20
M — *V*	1839.59	5	112.07	*H.S.*	367.91
L — *T* — *M*	784.40	5	47.78	*H.S.*	156.88
L — *T* — *V*	1031.43	25	62.83	*H.S.*	41.25
L — *M* — *V*	1509.51	25	91.96	*H.S.*	60.38
T — *M* — *V*	698.49	5	42.55	*H.S.*	139.69
L — *T* — *M* — *V* (Error)	426.22	25	25.96	0.50–0.30	17.04
Total	102054.21	143			

$V_{\varnothing} = 16.414.$

length of time in cold room was determined for the five inbred sweet corns. The choice of order of the polynomial regression showed that the simplest equation of the general form $y = a + bx$ was in all cases adequate. The actual values for these formulae are given in Table III and the slopes plotted in Figure 3. The slopes

TABLE III

Regresson Formulae For Sweet Corn Data	
Experimental Conditions	Regression Formulae
General Formula	$a - b\ (x - \bar{x})$
Pooled data	$175.76 - 49.08\ (x - \bar{x})$
(i) 50°F — soil	$39.04 - 15.74\ (x - \bar{x})$
(ii) 40°F — soil	$54.72 - 6.72\ (x - \bar{x})$
(iii) 50°F — Sand	$45.24 - 15.60\ (x - \bar{x})$
(iv) 40°F — Sand	$36.76 - 11.02\ (x - \bar{x})$

were tested for parallelity and heterogeneity by means of *b* coefficients (M a t h e r 1943). These tests showed that the slopes in the 40° and 50° soil tests are parallel and their means are not signifi-

cantly different, even though 40° results are higher than the 50° results throughout the experiment. The 40° and 50° sand regressions are just out of parallel and there is an initial difference between their

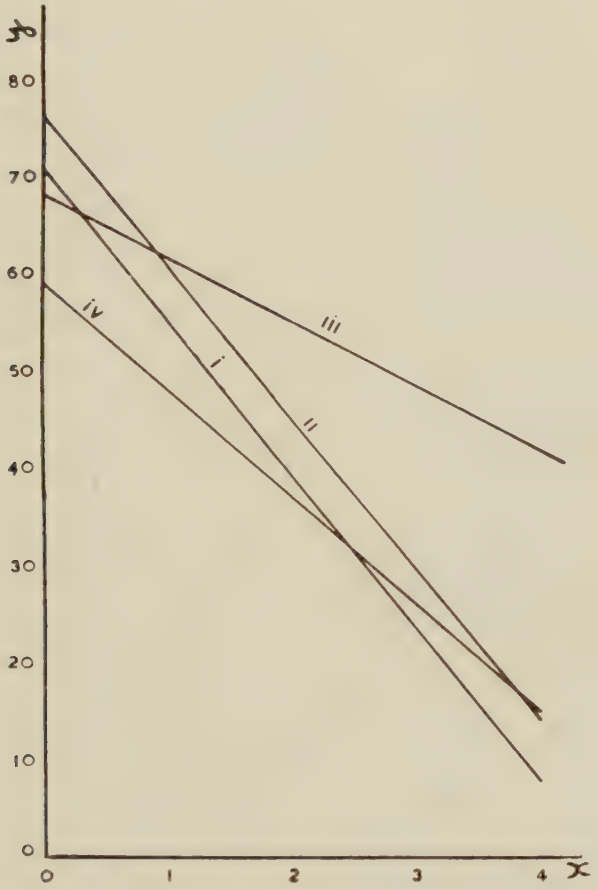

Fig. 3. Regression lines for the four regression formulae of germination (y) on length of time in cold room expressed in exponentials of log. Days (x).

Key: (i) 50°F cold rooms using soil.
(ii) 40°F ,, ,, ,, soil.
(iii) 50°F ,, ,, ,, sand.
(iv) 40°F ,, ,, ,, sand.

means. The 50° sand slope is significantly different from both soil slopes and the differences of their means is significant. The 40°

sand slope is significantly different from that for 50° soil but not from the 40° soil slope.

The theoretical values were not significantly different from actual values.

Discussion. Cold hardy lines of sweet corn may be selected by delaying emergence at low temperatures to allow pre-emergence killing off. The plant breeder using this method as a substitute for, or aid to, field selection would find that not less than 10 and not more than 16 days under these conditions are sufficient to permit germination of enough hardy plants from which to breed. Soil is preferable to sand for more rapid killing off. Using soil, a temperature of 50°F is preferable to 40°F. Regression lines indicate that the 50° soil method offers the most rapid means of discrimination.

Selection is determined by several factors influencing cold hardiness. These include resistance of germinating seeds to soil pathogens under poor conditions of germination. A large number of factors acting by themselves and simultaneously with others effect germination of sweet corn at low temperatures. The importance of their interactions is in accordance with the known complex behaviour of soil organisms (Garrett 1944). Temperature optima may be widely different not only for development of different parasities in the same host and for the development of the same parasite in different hosts but even for different phases of infection by one parasite in a single host. Maize, in contrast to wheat, is a species requiring high temperatures during germination and for successful stands. Therefore, as pointed out by Leach (1947), pre-emergence infection is most severe at temperatures relatively less favourable to the host than to the pathogen. In addition, the relative growth rates of both host and pathogen determine to a considerable degree the severity of pre-emergence infection at different temperatures. Still requiring investigation is what controls the resistance of seedlings to various parasites, so that while one strain is quickly killed off with a small amount of cold, others, e.g. C 4, can withstand long exposures to cold.

Acknowledgements. Thanks are due to Dr. W. R. Singleton for suggesting these investigations and for his interest and advice throughout the study. The author is also indebted to Dr. D. F. Jones for the facilities of the Genetics Department of the

Connecticut Agricultural Experiment Station, New Haven. He was then in receipt of a graduate grant from the Agricultural Research Council of Great Britain. He also wishes to thank Prof. K. Mather for statistical advice.

Summary

1. Five inbred lines of sweet corn and one hybrid dent corn were sown in soil and sand, and kept at temperatures of 50°F and 40°F, for different lengths of time. Their germination in a warm greenhouse was later determined.

2. The longer the seeds were kept in the cold the greater was the number of pre-emerged seedlings killed off.

3. The various strains reacted differently to cold treatment. The early inbred C 4 was most resistant to treatment and even germinated better than Conn 520; the endospermal allele *Su/su* is thus not so important as the selection of the strain for hardiness.

4. Genetical constitution, temperature, length of time in low temperature, and the nature of the germinating medium all significantly affected final germination. Also important were their first and second order interactions, especially that between temperature and medium.

5. Linear equations were fitted for final germination against length of time in the cold rooms using different media. Those for the 50°F, and 40°F treatments using soil were parallel while those of the 50°F and 40°F using sand were slightly out of parallel with each other. The differences in the regression lines are largely due to the germinating medium and to the reaction between this and the low temperature.

6. As an aid to plant breeding, the controlled cold treatment offers a method suitable for artificially selecting hardier strains of sweet corn.

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Plant and Soil II, no 1 October 1949

MINERAL NUTRITION IN RELATION TO THE BIOCHEMISTRY AND PHYSIOLOGY OF POTATOES

I. EFFECT OF NITROGEN, PHOSPHATE, POTASSIUM, MAGNESIUM AND COPPER NUTRITION ON THE TYROSINE CONTENT AND TYROSINASE ACTIVITY WITH PARTICULAR REFERENCE TO BLACKENING OF THE TUBERS

by E. G. MULDER *)

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1. Introduction.
2. Experimental methods.
3. Relation of the mineral nutrition of potato plants to the tyrosine-tyrosinase reaction and the occurrence of black discolorations in the tubers.
4. Tyrosine content of potato tubers grown under various conditions.
5. Tyrosinase activity of potato tubers in relation to mineral nutrition.
6. Discussion.
7. Summary.

1. *Introduction.* In the course of an investigation into the effect of the mineral nutrition of potato plants on the chemical composition of the tubers, a relation was found between the potassium supply to the plants and the content of free tyrosine in the tubers. A high tyrosine content was found to be associated with inadequate potassium supply. It is a well-known fact that under favourable conditions tyrosine can readily be converted into red and thereafter into black oxidation products by the enzyme tyrosinase which is naturally found in potato tubers [20]; an investigation was undertaken therefore to determine whether the blue-black discoloration which often appears in potassium-deficient potatoes, particularly when bruised by rough treatment, (Oortwijn Botjes en Verhoeven[23]), Oortwijn Botjes[24])), is due to the high tyrosine content.

Suggestions as to the role of the tyrosine-tyrosinase reaction in producing black discolorations in potato tubers, have been made by De Bruyn[5]) Merkenschlager[17]) and Hansen[11]).

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Neither tyrosine nor tyrosinase determinations were carried out by these investigators, however. According to de Bruyn the discolorations are due to injury of the cells without inactivation of the enzymes. Tubers or parts of tubers liable to blacken, were found to have a higher specific gravity than normal ones. Contrary to the findings of Oortwijn Botjes [24]) and Verhoeven [40]), she observed no clear relation between potassium supply and blackening. Hansen, however, also found that potassium-deficient potatoes were more prone to show discoloration than normal ones. Koblet [13]) found a negative correlation between K-content and blackening of the tubers in the variety Voran but not in Ackersegen.

In the literature much attention has been paid to the phenomenon of "blackening after cooking" of potato tubers but no data are available, concerning the relation between the discoloration of living and that of boiled potatoes. According to Smith, Nash and Dittman [34]) the latter kind of blackening is not found in raw tubers. The photograph of tubers, discoloured after cooking, shown by Rieman, Tottingham and McFarlane [27]) does not resemble the picture of blackening observed by the author in raw potassium-deficient tubers. It must be emphasized, however, that neither does this photograph agree with the description given by various American authors. According to them, blackening after cooking is found mainly at the stem end of the tubers ,just as in the tubers investigated by de Bruyn [5]), Verhoeven [40]), Oortwijn Botjes [24]) and Hansen [11]).

As to the cause of the blackening of boiled potatoes opinions are conflicting. In the first communications of Tottingham *et al.* [29]) [37]) it was shown that the protein of abnormal potatoes is less firmly condensed than that of white-cooking tubers, and consequently is more easily hydrolysed by dilute sodium hydroxide or by proteolytic enzymes. In abnormal tubers [30]) the accumulation of α-amino acids, particularly tyrosine, in combination with a more pronounced tyrosinase activity, was supposed to be responsible for the blackening upon boiling. Although Tottingham *et al* [37]) found somewhat increased contents of free α-amino nitrogen, tyrosine and tryptophane in abnormal potatoes, no relation between tyrosine content and degree of blackening after cooking was observed by Nash [19]).

In their early investigations Tottingham *et al.* found a general relation between discoloration after cooking and potassium supply [37]). In subsequent publications of the same authors, however, the occurrence of such a relation was denied [7]) [38]). Smith, Nash and Dittman [34]) also came to the conclusion that various levels of nitrogen, phosphorus, potassium and lime exert no consistent effect upon the incidence of blackening. The same is true of soil reaction, soil moisture, soil types and deficiencies of boron, copper, zinc, manganese and magnesium. Wallace and Wain [41]) state that blackening of cooked potatoes may be brought about by K-, P- and possibly Ca-deficiency.

According to Robison [28]) the black pigment is not melanin, for in the potatoes she investigated the colour disappeared rapidly at pH 3.0. She

suggested that the black colour results from the oxidation of a colourless ferrous compound which is formed upon boiling. Cowie[8]) also attributed a role to iron in the blackening which occurs after cooking, and stated that the discoloration is connected with a low potassium and a high nitrogen level in the soil.

Nutting[21]) [22]) suggested that the black pigment might be a loose complex of a metal and a yellow pigment normally present in the tuber. This suggestion is in agreement with the older finding of Tinkler[36]) and also of Mader and Mader[16]) that a high content of iron favours the blackening after cooking.

Wager[42]) apparently studied the same kind of blackening as did Robison, since he likewise observed that the discoloration disappeared upon treatment with acids.

When the present paper had been almost completed the author took note of an extensive study on the blackening of potatoes after cooking, published recently by Juul[12]). In agreement with Robison, Nutting and others, Juul comes to the conclusion that it is due to an iron compound. He advances the theory that the ferrous ions of the potato upon cooking combine with an *o*-dihydric phenol to give a colourless compound which is oxidized to the strongly coloured ferric compound on being exposed to the air. Since he isolated caffeic acid from the peel of potato tubers, he suggests that this is the *o*-dihydric phenol mainly responsible for the pigmentation. Since the colour intensity of Fe-compounds of *o*-dihydric phenols is clearly related to the pH, it is assumed by Juul that the discoloration of cooked potatoes depends to a large extent on the pH of these tubers. Considerable evidence is given as to the importance of the latter factor. In addition to the pH, other factors were found to affect the degree of discoloration viz. the content of *o*-dihydric phenols and the iron content. Increasing N/K ratio in the fertilizers supplied, which was found to increase the liability to blacken upon boiling, was correlated with a small but significant rise in pH and a considerable rise in *o*-diphenol content. Likewise, stem ends of potato tubers, which blacken more intensely than bud ends, were found to have a somewhat higher pH and a higher content of *o*-diphenols. No effect of the N/K manuring was found on the ferrous content of the tubers.

In some publications stress is laid on the importance of climatic factors in rendering potatoes liable to blacken after cooking. According to Smith *et al.*[34]) tubers maturing under relatively high temperatures do not blacken upon boiling. The severity of blackening increases as the mean temperature during the last months of the growing season becomes lower.

Tottingham *et al.*[38]), however, observed that discoloration after boiling is more evident in those summers which are hotter and drier than usual. In a subsequent paper[27]) it is stated that low temperatures during June and September and high temperatures during August produce the most severe effect.

Smith *et al.*[34]) found a relation between temperature during storage and blackening. Temperature treatment for some days at 104°C decreased

the amount of blackening to about one twentieth of that of untreated tubers. The pH of the treated tubers was found to change from 6.10 to 5.60. These results are easily understood when the investigations of J u u l are taken into consideration.

In a recent survey, B u r t o n [6]) concluded that the predisposition of potatoes to stem- end blackening after cooking may depend upon a great number of factors.

In the present investigation tyrosine content, tyrosinase activity and the blackening of potatoes were studied in relation to the supplies of potassium, nitrogen, phosphate, magnesium and copper. Special attention was paid to the relation between blackening in raw and that in boiled tubers. Furthermore an extensive stu dy was made of the tyrosine metabolism in potato plants and of the physiological importance of this amino acid in potatoes. The latter aspect of this research is still in progress and the results will be published in a subsequent paper.

2. *Experimental methods.* Two varieties of potatoes, the protein-rich Noordeling and the protein-poor Voran *), were grown on soils poor in one or two of the following nutrients: nitrogen, phosphate, potassium, magnesium and copper. By supplying different amounts of fertilizers, potatoes were obtained with all grades of nitrogen, phosphate, potassium, magnesium and copper deficiencies.

The tubers were harvested carefully to prevent bruising as much as possible. They were washed in a stream of tap water and stored in a cool room until analysed.

A n a l y t i c a l m e t h o d s.

For chemical analysis cylinders of about 1 cm diameter were taken longitudinally and transversely with a cork borer from about 20 tubers. In many cases heel and rose halves of the tubers were analysed separately. The cylinders were minced and 10 g of this material were placed in a porcelain mortar. Either 4 cc of glacial acetic acid or 5 cc of a 1% Na_2SO_3 solution were added in order to prevent the enzymatic oxidation of tyrosine. Both inhibitants give about the same values for free tyrosine (see Table 5) but the former has the disadvantage that it prevents the precipitation of water-soluble proteins when the filtrates are boiled. However, the mercury salts used in the tyrosine analysis precipitate these proteins, so that no interference occurs in the determination of the soluble tyrosine. When other amino acids are to be determined, as for instance tryptophane or arginine, the acetic-acid-treated samples give too high values. In this case

*) Certified seed stocks were used in all experiments.

the procedure is as follows. After grinding the weighed samples, the pulp is transferred to a Büchner funnel and filtered. The filtrates, which contain the soluble proteins, amino acids, amides etc., are boiled for some minutes, after adding a few drops of acetic acid and a knife point of sodium chloride. The proteins precipitate and after cooling are removed by centrifuging. The precipitate is washed 3 times with small amounts of distilled water containing a trace of acetic acid, which after separation of the proteins is added to the bulk of centrifugate. A further washing with alcohol needs to be carried out when the proteins are to be hydrolysed.

The tyrosine in the clear solution *) is determined by L u g g's method [15]). The procedure may be described briefly. To 20 cc of the test solution contained in a 50 cc centrifuge tube is added sufficient 5 N H_2SO_4 solution to bring it to pH 0.3. 5 cc of solution *A* (see below) are added and the tube is maintained at 60–65° in a water-bath for 30 minutes. It is then cooled for 1 h in running tap water and after centrifuging, the clear liquid is poured into a graduated flask. 10 cc of *B* are run into the centrifuge tube, the precipitate is stirred and the contents are again centrifuged. The clear liquid is poured into the flask and the contents are diluted to 49 cc with H_2O. Then 1 cc of a 1 M $NaNO_2$ solution is run slowly into each flask so as to flood on top, and the flasks are shaken as soon as possible thereafter.A red colour develops and colorimetric readings can be made after 3 minutes' incubation at a temperature not below 20° (Filter 520 mμ).

Reagents: Solution A: 75 g $HgSO_4$, 55 g $HgCl_2$, and 70 g Na_2SO^4 are dissolved in 850 cc water plus 125 g 98% H_2SO_4 and diluted to 1 l. Solution B: A certain volume of A is diluted with an equal volume of 1 N H_2SO_4.

Isolation of tyrosine.

5 kg of potatoes were ground in a mill. The pulp was immediately treated with 50 cc of 30 per cent acetic acid and then diluted with a small amount of water. After squeezing the solutes through a cloth the residue was ground in a mortar while adding a further 20 cc of acetic acid. After removal of the solutes this operation was repeated twice. The solution was heated above 80°C for 10 minutes and after cooling was filtered. To the total volume of solution, about 4 l, was added an excess of neutral lead acetate. The precipitated proteins were removed on a Büchner funnel, and the excess of lead in the filtrate precipitated with H_2S. Lead sulphide was removed by filtration and the clear filtrate freed from hydrogen sulphide by means of an air stream.

*) Treatment of this solution with ether at pH 1, which removes *p*-hydroxybenzoic acid and ordinary phenol, and with toluene at pH 6–7, which removes indole and skatole, was not undertaken since practically no effect of these treatments was found on the tyrosine content of potato tubers. The very slight reduction in estimated tyrosine content which occurred in some cases after these treatments may just as well be attributed to losses in tyrosine.

3, 4-dihydroxyphenylalanine which may occur in potato tubers does not interfere with the tyrosine determination.

In this solution tyrosine was precipitated by adding 1 l of a 10 per cent $Hg(NO_3)_2$ solution. This latter was prepared by dissolving $Hg(NO_3)_2$ in water with addition of concentrated nitric acid. Then sodium hydroxide was added in just insufficient quantity to precipitate HgO.

Upon the addition of the weakly acid $Hg(NO_3)_2$ solution a bulky precipitate was formed. This precipitate was separated by filtration and dissolved in water acidified with 2 cc of concentrated H_2SO_4. Hydrogen sulphide was introduced to decompose the mercury-tyrosine compound; HgS was removed by filtration and H_2S by a stream of air.

The tyrosine-containing solution was neutralized and then concentrated on a water-bath at a temperature not exceeding 70°C. When the volume had been reduced to about 1 l, further concentration was effected in vacuo at about 40°C. In the concentrated solution tyrosine crystallized. It was recrystallized twice from distilled water, collected, dried and analysed for carbon, hydrogen and nitrogen.

Protein and soluble non-protein nitrogen.

Protein and soluble non-protein nitrogen were determined as follows: 10 g of the average samples mentioned on page 62 are ground in a porcelain mortar and transferred to 100 cc flasks, using about 50 cc H_2O. The solutions are heated in a water-bath and the temperature maintained above 90° C for 15 minutes. The coagulated starch is then brought into solution by adding 10 cc of a 1% diastase solution and incubating for a sufficient time for all the starch to be hydrolysed (about 6 h at 46°C). The flasks are shaken occasionally during this period. 1 cc of toluene is added initially and if necessary is renewed during the incubation period. After cooling, 12,5 cc of a 20% trichloracetic-acid solution are added to precipitate the protein. The latter is separated on a Büchner funnel and washed three times with a 1% trichloracetic-acid solution. Residues as well as filtrates are analysed for nitrogen according to the Kjeldahl-Lauro method.

o-Dihydric phenols.

For the determination of *o*-dihydric phenols the following method was used: 10 g of the above samples are rinsed with H_2O, transferred to a porcelain mortar together with 4 cc acetic acid, and ground. The pulp is transferred to a Büchner funnel and filtered. The residue is washed three times with small amounts of distilled H_2O. The filtrate is made up to volume and *o*-diphenols determined by the method of Arnow [1]: To 1 cc of the solution in a test tube graduated to 10 cc the following reagents are added: 1 cc of 0.5 N HCl, 1 cc of nitrite-molybdate reagent (10 g of $NaNO_2$ and 10 g of Na_2MoO_4 in 100 cc of distilled H_2O), 2 cc of 1 N NaOH and enough distilled water to make up the volume to 10 cc. The resulting red colour is determined in a colorimeter using a filter of 460 mμ. 3,4-Dihydroxyphenylalanine (dopa) is used as a standard. Catechol gives a similar colour. According to Arnow resorcinol gives a deep brown colour and pyrogallol a deep red brown.

Tyrosinase activity.

The tyrosinase activity of potato tubers was determined as follows: 5 or 10 g of tissue are ground in a porcelain mortar and transferred to a 200 cc scrubbing flask. A solution containing 25 mg of l-tyrosine and 0.5 cc of toluene is added and the volume made up to 100 cc. The flask is incubated at 20°C and air bubbled through the solution. Samples of 10 cc are pipetted at the beginning of the experiment and subsequently at intervals. In these samples the enzyme activity is stopped by adding 4 cc of glacial acetic acid. The solutions are made up to 50 cc and tyrosine is determined by the above-mentioned method.

3. *Relation of the mineral nutrition of potato plants to the tyrosine-tyrosinase reaction and the occurrence of black discolorations in the tubers.* Although many discolorations in potato tubers are due to the activity of viruses, bacteria or fungi, it can be stated with certainty that the greyish-brown or bluish-black areas which occur in tubers of potassium-deficient plants have nothing to do with an infectious disease. This can be concluded from the fact that potassium-deficient tubers which have been handled very carefully, so that they are practically free from discoloration, may show great areas of blackened tissue a few hours after being shaken for a short time.

When potato tubers differing in their mineral nutrition were investigated, it was consistently found that those from plants with an inadequate potassium supply showed greyish-brown or blue-black discolorations. The severity of potassium-deficiency symptoms on the leaves provided a measure of the amount of discoloured tissue in the tubers.

Although very careful harvesting and transportation of the tubers prevented to a considerable extent the occurrence of coloured lesions, it was nevertheless impossible to obtain normal tubers of Noordeling from plants displaying heavy symptoms of K-deficiency. Apparently the pressure which occurs in growing tubers sufficed here to bring about the circumstances under which discoloration takes place.

When the tubers were left unmoved for some months after harvesting, black and grey-brown tissue was found. These discolorations occurred as small brownish spots situated between the surface and the vascular ring and also as larger areas in the interior of the tuber. They were practically always confined to the heel half of the potato.

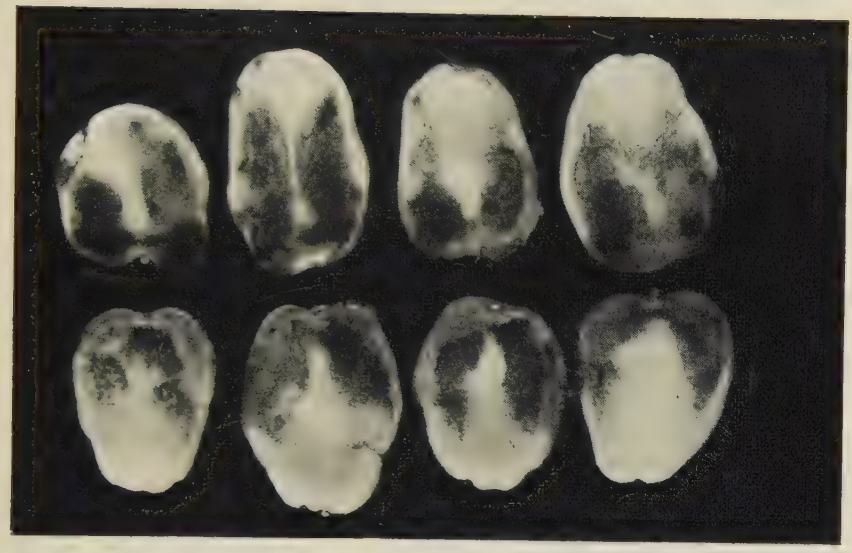
When such tubers were shaken for 5 minutes in a bottle, or else subjected to rough transportation, great areas of white tissue at the

stem end turned blue black within a few hours. The same was true, though to a lesser degree, when the tubers were cut with a knife, washed with tap water and exposed to the air. Apparently the tension resulting from the cutting had more or less the same effect as the shaking. The tissue first turned reddish and after some hours darkened to bluish-black (see Plate I and II).

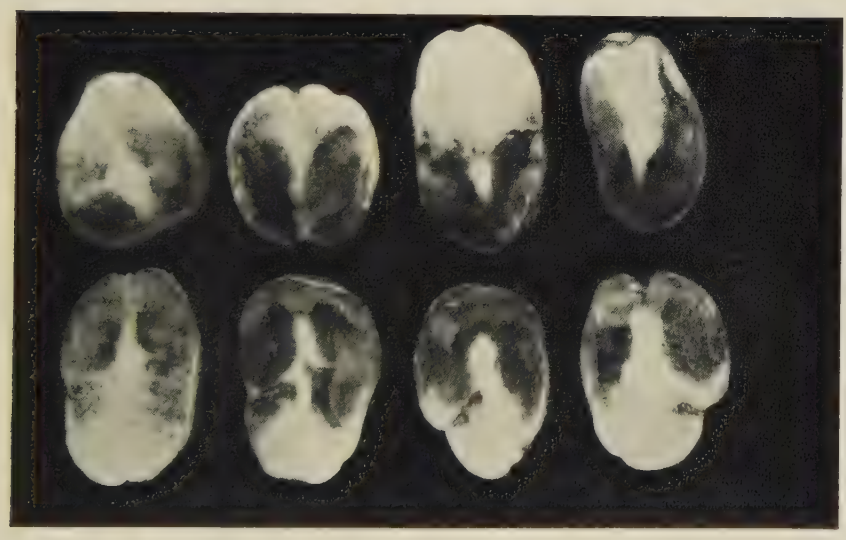
a. Role of enzymatic reactions in the blackening of potatoes. The fact that the blackening appeared after shaking or cutting the tubers made it probable that an enzymatic reaction is responsible. That this is true, was shown by the following experiment. Transverse slices of tubers, about 2–3 mm thick, were heated for a few minutes at their centres by a small flame and then exposed to the air. After some hours a picture like that shown on Plate III was obtained. In the centre, where enzymes as well as cells were killed by the flame, no discoloration of the tissue was to be seen. In a ring about 5 mm wide surrounding this white tissue the colour turned red and afterwards black. Apparently in this annulus the temperature had been high enough to kill the cells, but the enzymes had remained active. In the tissue outside the black ring again no discoloration was to be seen, owing to the fact that the cells were undamaged.

Although the potassium-deficient tubers showed much greater discoloration than those with a normal potassium supply, a distinct ring developed likewise in the slices of the latter. Therefore the substances which give rise to the black colour are present also in normal tubers though in considerably smaller amounts.

In order to gain further information concerning the temperatures at which the cells, and the enzymes responsible for the blackening, are destroyed, the following experiment was carried out. Slices of potassium-deficient tubers, 2–3 mm thick, were immersed for 20 minutes in water-baths at different temperatures. They were then exposed to the air and some hours later the degree of blackening was ascertained. An optimal discoloration was obtained at 55°C. At 60° the blackening was somewhat less intense owing to the onset of inactivation of the enzymes. At 70° enzyme activity was much more reduced and at 80° no discoloration at all took place. Below 55° on the other hand blackening was less intense because of reduced injury to the cells. After treatment at 40° for 20 minutes considerable

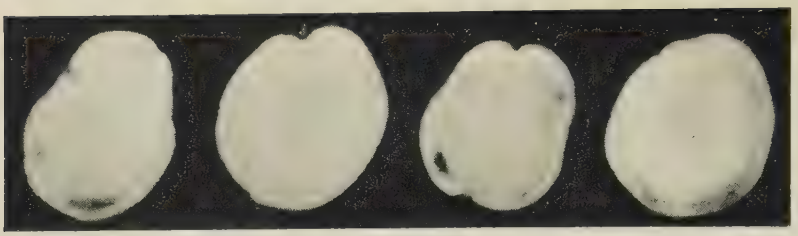


A

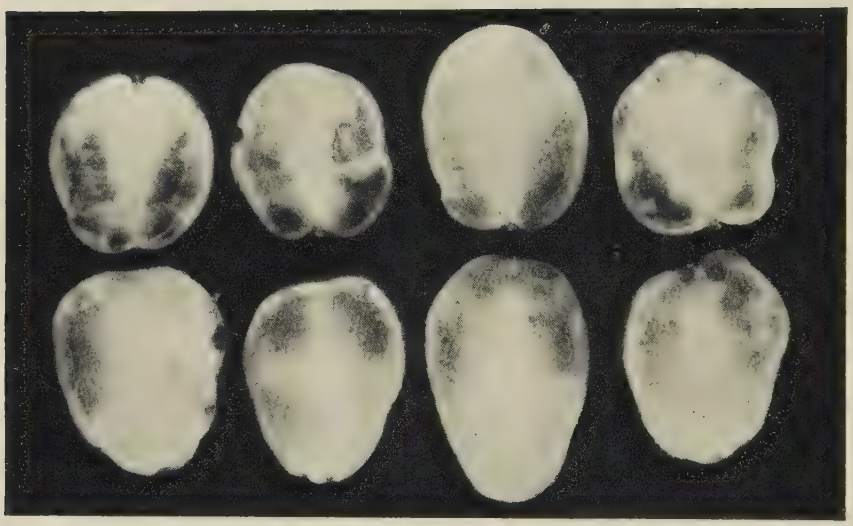


B

Plate I. Tubers from Noordeling potatoes with heavy symptoms of potassium deficiency (Exp. 589, 1948). Stored from October until February, cut longitudinally, rinsed with tap water and exposed to air overnight. Stem ends pointing towards the middles of the photographs. A: no nitrogenous fertilizers applied. B: supplied with nitrogen at the rate of 200 kg per ha. Upper row of both A and B shaken for five minutes in a bottle before halving. Black tissue mainly confined to stem ends of the tubers. Yield data are given in Table 6.



A



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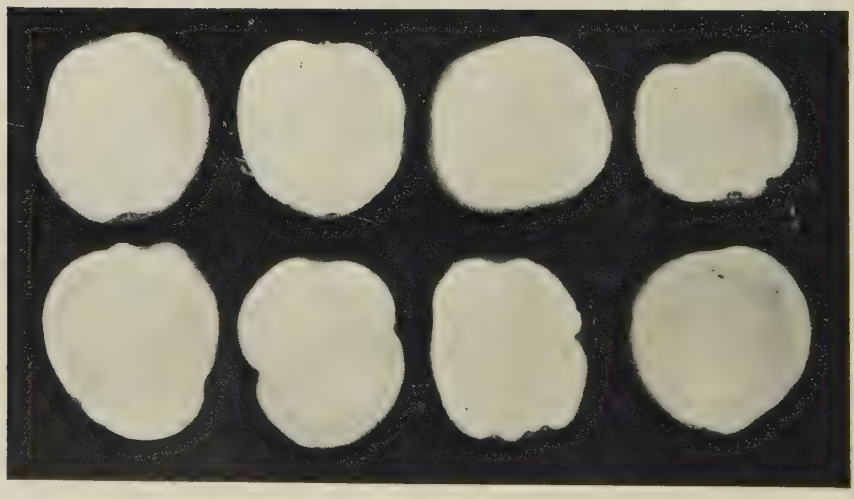


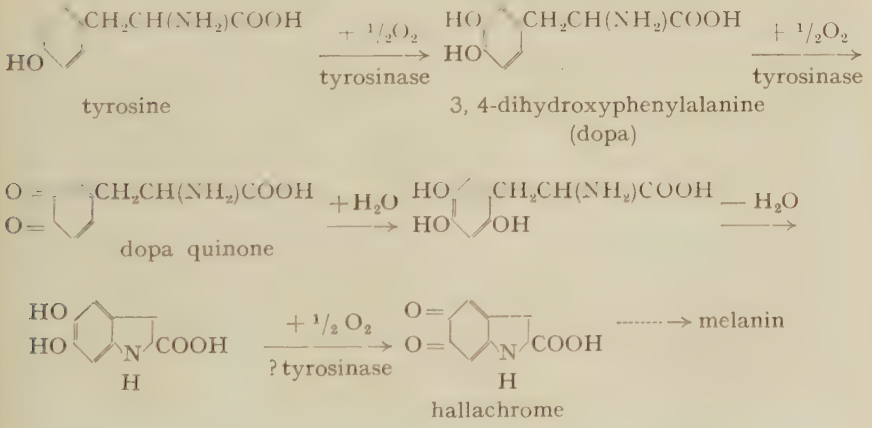
C

Plate II. Tubers from Noordeling potatoes. (Exp. 589, 1948). Stored from October until February, cut longitudinally and rinsed with tap water. Stem ends pointing towards the middles of the photographs. A and B from plants dressed with potassium at the rate of 100 kg K_2O per ha and with nitrogen at 200 kg per ha. A photographed immediately after halving, B after exposure to air overnight. C: tubers from plants dressed with K at the rate of 600 kg K_2O per ha and N at 200 kg per ha. Upper row of both B and C shaken for five minutes before halving.

blackening still took place, but a temperature of 30° did not affect the cells.

In a further experiment potato slices were exposed to toluene vapour. It is well-known that in such an atmosphere cells are killed but the enzymes remain active (Boas and Merkenschlager [2]). In conformity with this, at first a reddish and later a bluish-black discoloration of the tissues was produced as in the bruised tubers. In K-deficient tubers the discoloration following toluene treatment was again much more intense than in normal ones.

The results of these experiments clearly show that the blackening of bruised potato tubers is due to an enzymatic reaction. It is a well-known fact that black discolorations in both animal and plant tissues may be brought about by an enzymatic formation of melanin from tyrosine or other mono- or dihydric phenols. In this reaction tyrosine is oxidized by tyrosinase (monophenol oxidase, polyphenol oxidase, catechol oxidase) to 3, 4-dihydroxyphenolylalanine (dopa). This latter compound is then further oxidized to the corresponding quinone which, presumably after spontaneous ring closure, is converted into hallachrome, a red compound. Subsequent changes, which include polymerization, lead to the formation from hallachrome of the blue-black pigment melanin [3] [26]).



Since the blackening of bruised potato tissues proceeds also through a red stage, it has been supposed that the tyrosinase reaction is responsible for this phenomenon. Suggestions along the same

lines have been made by de Bruyn[5]) and Hansen[11]) but as far as the author is aware without presenting any convincing evidence.

b. The role of the tyrosine-tyrosinase reaction. That the tyrosine-tyrosinase reaction is indeed responsible for the black discolorations in raw potassium-deficient potato tubers may be concluded from the following facts.

1. Potassium-deficient tubers which are very prone to discolour have a free-tyrosine content three or four times as high as that of tubers with a normal potassium supply which do not darken (see section 4).

2. The variety Noordeling, which is much more liable to blacken than the variety Voran, has a tyrosine content about twice as high as that of the latter.

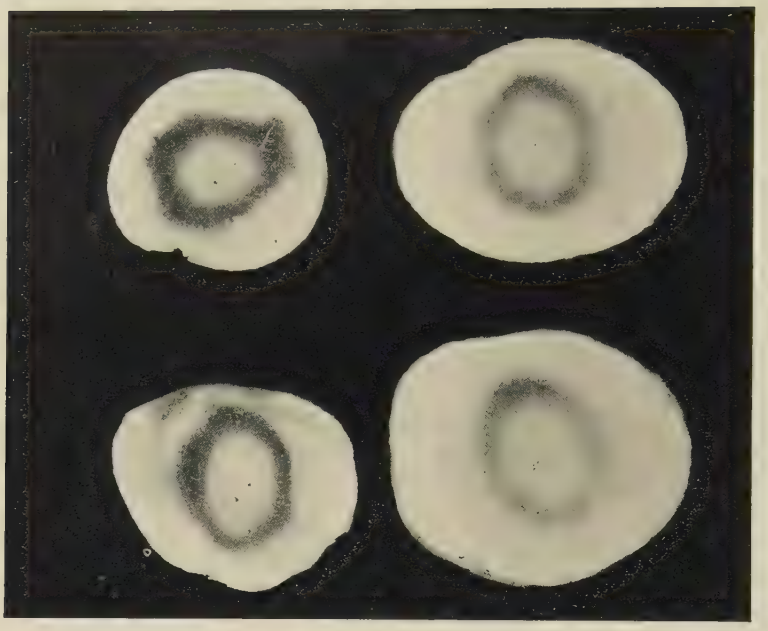
3. A pulp or an extract of potassium-deficient tubers exposed to the air turns at first red and then reddish-brown, black-brown and finally black, but tubers with a normal potassium nutrition give only a greyish-brown pulp or extract under the same conditions. However, the addition of a few mg of tyrosine to 10 g of pulped tuber tissue results in a coloration equal to that developing spontaneously in pulped potassium-deficient tubers (see p. 72).

4. Potatoes grown on a soil poor in copper as well as in potassium are far less prone to blacken than those grown on a similar soil dressed with copper sulphate. This is accounted for by the fact that tyrosinase is a copper-containing enzyme[14]). As will be shown in section 5, the tyrosinase activity of the copper-deficient tubers may be less than one tenth of that of tubers dressed with copper sulphate.

5. No discoloration of potassium-deficient tubers takes place when the uptake of oxygen is prevented e.g. by adding a small amount of Na_2SO_3 or by immersing the tissues in water. As the tyrosine-tyrosinase reaction is an oxidation, this result is readily understandable.

c. Resistance of K-deficient tubers to bruising. Although the high tyrosine content of potassium-deficient potato tubers is of great importance in the blackening of the tissues, it is not the sole determining factor. This was concluded from the fact that tubers well-supplied with potassium, which after rough

A



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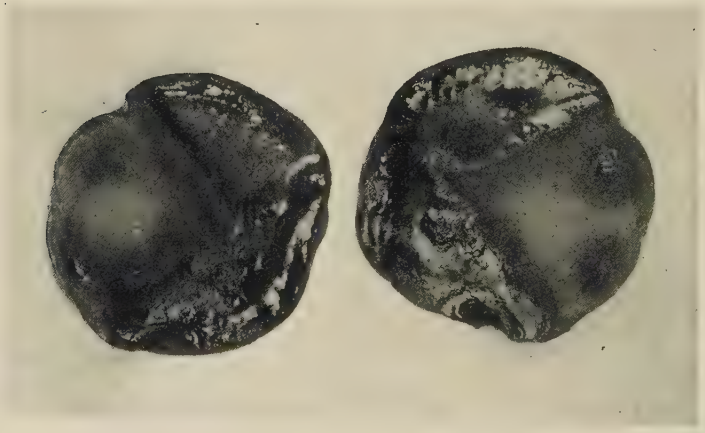

C

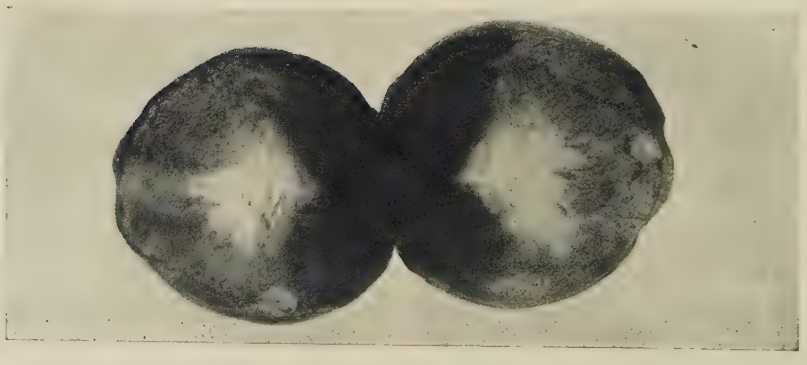

Plate III. A. Slices of K-deficient (left) and normal (right) Noordeling potatoes, heated in the centre with a small flame and thereafter exposed to the air for a few hours. B. Potassium-deficient tubers, shaken for five minutes in a bottle, halved, rinsed with tap water and inoculated with *Bact. prodigiosum*. Development of this bacterium has taken place on black tissue only. C as B, uninoculated.

treatment developed scarcely any black discoloration, nevertheless showed a rather weak but clearly perceptible coloured ring upon being heated with a small flame (see Plate III). A similar discoloration was observed also when slices of normal tubers were exposed to toluene vapour, or when whole tubers were bruised by extremely heavy shaking. Although the resulting colour was much weaker than that developed in the potassium-deficient tuber slices, nevertheless the small amount of tyrosine in the former was apparently sufficient to produce this discoloration.

Another fact pointing in the same direction is that the heel halves of potassium-deficient tubers, which become much more blackened after rough treatment than do the rose halves (see Plate 1 and 2), contained only 20–40% more tyrosine, while the tyrosinase activity in both parts was about equal (see section 5) *). Apparently another factor besides the high tyrosine content must be responsible for the extreme liability of K-deficient tubers to discolour.

As the tyrosine-tyrosinase reaction can take place only when tyrosine, tyrosinase and oxygen all come together, the obvious assumption is that these conditions are much more easily fulfilled in potassium-deficient than in normal tubers. Potassium-deficient tissue, particularly that of the heel halves, is apparently much more liable to bruise than is that of tubers with an abundant potassium nutrition. The following experiment demonstrates the truth of this assumption. Potassium-deficient and normal tubers were shaken gently for some minutes in a bottle. They were then halved longitudinally. The cut surface of one half, after being washed with tap water, was inoculated with a culture of the saprophtic *Bacterium prodigiosum*; the other half remained uninoculated. As *Bact. prodigiosum* is unable to develop on living potato tissue, its growth indicates that the cells are dead. It appeared that an abundant development of *Bact. prodigiosum* took place only on those parts of the K-deficient tubers where a black discoloration was to be seen in the corresponding uninoculated halves (compare Plate III, *B* and *C*). On the tubers with a normal potassium supply, which after shaking

*) That the oxygen supply does not differ in the two halves may be concluded from the fact that H_2S penetrates in to both at the same speed. This was shown by exposing K-deficient and normal tubers for different periods of time ($^1/_4$, 2 and 4 h) to an atmosphere of H_2S. Penetration of the gas was determined by halving the tubers longitudinally and treating the cut surface with a solution of Pb-acetate.

showed no discoloration, *Bact. prodigiosum* did not grow. When, however, these tubers were boiled for a few minutes, before inoculation an abundant growth took place over their whole cut surface. From this result the conclusion may be drawn that blackening of potato tissue only takes place after death of the cells.

The fact that the tyrosine-tyrosinase reaction proceeds only when the cells are killed, does not necessarily mean that in the intact cells tyrosine and tyrosinase are located apart e.g. tyrosine in the cell sap and tyrosinase in the protoplasm *). It may be explained just as well on the assumption that in the living cell a powerful reduction system prevents the oxidation of tyrosine. Injury to the cells may destroy this reduction system, with the result that the oxidation can proceed. *In vitro* such a system was easily realized by adding a few mg of ascorbic acid to a potato pulp containing dopa and tyrosinase. As long as ascorbic acid was present no oxidation of dopa took place, even though air was bubbled through the solution. Ascorbic acid was oxidized with great speed, however, and as soon as it was completely converted, oxidation of dopa began.

The following experiment gives some information about the localization of tyrosinase in the cells of potato tubers.

10 g samples of K-deficient tissue were ground and the pulp transferred to a Büchner funnel. The solutes were separated and the residue washed thoroughly with about 45 cc H_2O. Both the filtrates and the cell residues were then transferred to 300 cc wide-mouthed Erlenmeyer flasks containing. 20 mg of tyrosine in 50 cc water. The filtrate from one particular sample (C) was made up to 50 cc and of this solution 2.5, 5, 10 and 20 cc portions were separately employed.

After adding 1 cc toluene, the flasks were incubated for 15 h at 28°C. Tyrosinase activity was then stopped by adding 4 cc of acetic acid, and the amount of tyrosine determined. Table I gives the results of this experiment.

It appears that the tyrosinase activity is found for the most part in the soluble fraction of the potato tuber.

Summarizing the results from the above-mentioned experiments, it may be concluded that two factors are responsible for the considerable tendency of potassium-deficient potatoes to display black

*) Szent-Györgyi and Victorisz[85]) concluded from the results of their experiments that polyphenol oxydase and phenolic compounds are located apart in the potato cell.

TABLE I

Tyrosinase activity of solutes and of cell residues of potassium-deficient potatoes

Sample	mg tyrosine oxidized in 15 h
A 1, residue of 10 g tissue	6.7
B 1, ,, ,, 10 g ,,	8.2
C 1, ,, ,, 10 g ,,	8.4
A 2, filtrate of A 1	12.6 *)
B 2, ,, ,, B 1	13 4 *)
C 2, *a*) 1/20 of filtrate of C 1	2.6
b) 1/10 ,, ,, ,, C 1	3.8
c) 1/5 ,, ,, ,, C 1	7.3
d) 1/2.5 ,, ,, ,, C 1	10.5 *)

*) The fact that increasing amounts of filtrate did not result in proportional oxidation of tyrosine is probably explained by lack of oxygen with the higher amounts of filtrates, since in this experiment no air was bubbled through the solutions.

discoloration viz. the high content of free tyrosine and the liability of the cells to bruise. A relatively slight pressure suffices to kill large complexes of cells with the result that tyrosine can be oxidized by tyrosinase activity first to a red compound and then to black oxidation products.

As to the cause of the increased liability of the cells of potassium-deficient tubers to bruise the following may be stated. De Bruyn[5]) found a relation between the tendency of potato tubers to develop discoloration and the specific gravity, in the sense that tubers or parts of tubers much prone to discolour had a higher S.G. than tubers or parts of tubers with little such tendency. This might mean that the water relations of the cells are concerned in the question of their liability to bruise. Similar results were obtained by Oortwijn Botjes and Verhoeven[23]) who found that "slack" tubers (i.e. tubers with a low water content) were much more prone to blacken after bruising than similar tubers whose water content had been raised before this treatment. These results are in good agreement with the fact that in spring when the water content of the tubers has decreased, their tendency to blacken is considerably more pronounced than in autumn. Apparently a high turgor renders the cells less liable to injury from shaking or pressure. Since the stem halves of potassium-deficient tubers are relatively low in water content, and when supplied with water absorb it to a considerably less extent than the bud ends or than the stem ends of normal tubers,

this may explain, at least partly, the greater liability to injury of their tissues.

d. Role of *o*-dihydric phenols in the blackening of potatoes. On page 68 it was stated that pulped potatoes with a normal potassium supply, whose tyrosine content had been brought up to the same level as that in pulped potassium-deficient tubers, developed a similar colour to that shown by the latter. This, however, was true only after exposure of the ground tissues to the air for a number of hours. When the discoloration was followed from the beginning, it was apparent that the potassium-deficient pulp reddened more rapidly than a normal one adjusted to the same tyrosine level. This was particularly the case when ground tissue from the cortical region of the stem end of potassium-deficient tubers was compared with a pulp from tissue inside the vascular ring of the bud end of normal tubers.

Since with pure preparations of tyrosinase the initial oxidation of tyrosine is strongly activated by the addition of a very small amount of an *o*-dihydric phenol (e.g. 3, 4-dihydroxyphenylalanine, (Pugh [25])), it was thought possible that this latter compound might be present in K-deficient tissue but lacking in normal tubers. No effect of the addition of a trace of dopa on the discoloration of a pulp from normal tubers supplied with tyrosine was observed, however. Determinations of *o*-diphenolic compounds showed that considerable amounts were present in potato tubers, especially in the cortical region (see Table XVII). Potassium-deficient tubers contained about twice as much as those with a normal potassium supply (see Table XVIII).

Since the oxidation of an *o*-dihydric phenol by tyrosinase proceeds much more rapidly than that of a monophenol, it must be concluded that the difference in velocity of discoloration of ground potato tissues with equal tyrosine contents but different *o*-diphenol contents is due to the oxidation of *o*-diphenol itself. That this assumption is correct, was demonstrated by adjusting both the tyrosine and the *o*-diphenol contents of normal pulp to their respective levels in K-deficient tissue. Discoloration then took place with the same velocity in both pulpes. Although that of normal tissue, to which dopa had been added, showed initially a somewhat brighter red colour, the ultimate colours attained after some hours were alike.

It may be asked whether the content of *o*-diphenols rather than tyrosine determines the amount of blackening of bruised potato tubers. In general it will make little difference finally whether the discoloration takes place very rapidly (dopa) or more slowly (tyrosine), hence it may be concluded, that tyrosine and *o*-diphenol are of equal importance in the production of black tissues in raw tubers. When, however, the enzymatic reactions can proceed only for a very short time (blackening after boiling) the *o*-diphenol content may be the more important.

e. Relation between blackening in raw and boiled potatoes. As is shown in section 1 various opinions prevail in the literature as to the cause and control of the so-called "blackening after cooking" of potato tubers. These opinions are so conflicting that it is highly probable the phenomena described are due to different causes. The fact that extensive descriptions of the symptoms observed, and of the nutritional and other environmental conditions under which the potatoes were grown, are missing in many publications, makes comparison between the various investigations often difficult.

It was not the author's intention to investigate all the factors mentioned in the literature as causing blackening in cooked potatoes. The behaviour upon boiling was investigated of only those tubers which in the experiments mentioned above were tested for discoloration following bruising.

Effect of the potassium supply. Noordeling potatoes grown on plots with different potassium dressings (Exp. 589) were peeled, transferred to enamelled pans containing cold tap water and cooked. They then were exposed to the air for half an hour and compared with similar samples which had been left uncooked but, after being halved longitudinally, had been exposed to the air overnight. Tubers from plants with heavy symptoms of K-deficiency, which before boiling already showed relatively large areas of blue-black tissue, retained their black colour upon boiling. Those from plants with less heavy symptoms of K-deficiency, although practically free from any discoloration before boiling, showed comparatively large areas of pale bluish-green tissue at the stem end when cooked. Tubers from plots with a moderate potassium supply became slight-

ly discoloured while those from plants with a heavy potassium dressing remained uncoloured.

When the cooked potatoes were compared with the corresponding raw ones which had been exposed to the air overnight, it appeared that the discoloration of the former was confined mainly to those areas which in the raw potatoes had blackened. Besides this stem-end discoloration a distinct bluish-green colour had developed in a few cell layers just beneath the peel (periderm) of the cooked potassium-deficient tubers. The latter discoloration was particularly evident when the peel (periderm) was removed after boiling and the tubers were exposed to the air for some time.

These results demonstrate that discoloration of boiled potatoes may be due to potassium deficiency. Apparently those factors which promote blackening in raw potatoes (high tyrosine and *o*-diphenol contents, tendency to bruise easily), also favour discoloration after cooking.

As to the nature of the coloured compound in cooked potatoes two views prevail in the literature, viz. 1. the pigment is melanin, 2. the pigment is not melanin but a complex containing a metal, presumably iron, and an organic compound (N u t t i n g [22]), a complex of iron and caffeic acid (J u u l) [12]), or merely black iron oxide (R o b i s o n [28])).

To decide whether the pigment is melanin or an iron compound, the coloured tissue may be treated with acetic acid. Thereupon melanin is unchanged, but if the colour is due to an iron compound it will disappear (R o b i s o n [28]), see also W a g e r [42])).

When the potatoes mentioned above were treated with acetic acid, the bluish-black areas of the potassium-deficient tubers which were already present before boiling remained unchanged; on the other hand the more bluish-green areas at the stem end of these tubers, which were uncoloured before boiling, together with the bluish-green surface layer just beneath the peel, disappeared completely.

These results indicate that both melanin and an iron compound may be concerned in the blackening of cooked potatoes. Not only the formation of melanin, but that of the other type of discoloration also is stimulated by K-deficiency. Further experiments were carried out to elucidate the part played by each of these substances in blackening after cooking.

Role of enzymatic processes in producing discoloration after cooking. When melanin is the cause of the discoloration upon cooking, it is to be expected that the latter will be most intense if the temperature in the tissue reaches the boiling-point slowly. That this is so was shown in the following manner. Transverse slices from uncoloured potassium-deficient and normal tubers, about 3 mm thick, were placed into boiling water immediately after cutting and cooked for some minutes. They were then exposed to the air. A similar set of slices was maintained at 55°C for 20 minutes, exposed to the air for about one hour, then cooked for some minutes and again exposed to the air for half an hour. A considerable development of bluish-black discoloration occurred in the latter case as a result of the prolonged period of enzymatic activity. The potassium-deficient slices were more intensely coloured than the normal ones, owing to their higher tyrosine and *o*-diphenol contents. The K-deficient slices cooked immediately after cutting showed a pale green-blue dicoloration at the stem ends and in a thin layer beneath the peel. Those from normal tubers were uncoloured.

It should be borne in mind, however, that normally the time during which tyrosinase can exert its activity is very limited viz. that between the damaging of the cells and the inactivation of the enzymes. Since the oxidation of tyrosine proceeds much more slowly than that of *o*-diphenols, it is to be expected that the content of the latter rather than that of tyrosine will provide the substrate for melanin formation in cooked potatoes.

In order to study the behaviour of tyrosine and dopa in potato tissue after cooking, tests were made with pulps and extracts of both potassium-deficient and normal tubers. Samples of Noordeling were ground and the pulp cooked. After grinding, the K-deficient pulp showed considerable reddening in contrast to that of normal tubers which coloured only slightly. Upon boiling the pulps, their red colour disappeared completely, but after cooling they turned black to an extent dependant on the original degree of reddening. Apparently the red pigment formed from dopa which disappears during the boiling is afterwards converted very quickly into melanin without the aid of enzymes.

When 1 cc of a 2 per cent Na_2SO_3 solution was added to the red-coloured pulp after boiling and decolourizing it, no discoloration took

place, indicating that the conversion of the decolourized red pigment into the bluish-black pigment is an oxidation reaction.

In a second set of tests use was made of extracts of normal tubers (20 g of tissue were ground and filtered; the filtrate, containing the greater part of the tyrosinase, was made up to 100 cc). 5 cc portions of these solutions, which showed a pale red colour, were pipetted into 20 cc tubes and 1 mg of dopa in 2.5 cc H_2O added. Control tubes were given 2.5 cc water. 15 seconds after the addition of dopa, when the solutions had already turned red, the tubes were brought to boiling point as quickly as possible. As this temperature was approached the solutions decolourized completely. After boiling for a few seconds the tubes were exposed to the air. Within half an hour the dopa-containing solutions developed a bluish-black colour, in contrast to the control solutions which became only slightly coloured.

The oxidation of dopa to the red pigment in potato extract took place with such velocity that heating a solution to boiling immediately after the addition of dopa did not prevent considerable reddening owing to the activety of tyrosinase before its inactivation.

Since in the above solutions the time was very limited during which enzymatic conversion of dopa to the red pigment could proceed, it appeared plausible that during the cooking of potatoes oxidation of dopa to the red intermediate should take place before tyrosinase is inactivated.

No red discoloration was observed, however, either of peeled K-deficient tubers or of tuber slices, at any moment during their cooking in boiling water. Apparently under these circumstances the oxygen supply is the limiting factor, since tubers whose cooking was interrupted, so that although the cells were killed the enzymes remained active, turned red after a few seconds' exposure to the air.

It must be concluded that melanin is responsible for blackening after cooking only in those cases in which conversion of tyrosine or dopa to red or black oxidation products has taken place before boiling. This may happen when potassium-deficient potatoes are bruised or when sodium chloride is added to the peeled raw tubers. In the latter case the cells are injured by plasmolysis and reddening takes place within a short time. In those cases, however, in which no coloration is visible before boiling, the blackening after cooking must be attributed to non-enzymatic processes, and presumably to the formation of an iron compound. This view is in harmony with the

fact that the latter type of discoloration occurs in slices which immediately after cutting are immersed in boiling water for 5 minutes and then exposed to the air for half an hour. Upon treatment with acetic acid the colour disappears completely (see p. 74). This type of discoloration also has been studied in some detail.

Non-enzymatic discoloration of potatoes after cooking.

As is stated above, the non-enzymatic discoloration of cooked potatoes is attributed by Robison [28]) to the formation during cooking of aferrous compound, which upon exposure to the air is oxidized to a ferric compound. This conclusion was reached because a marked correlation was found between iron content and the incidence of blackening in tubers drawn from the same sample. The correlation between tubers drawn from different samples was less clear. Non-blackening tubers could be made to blacken by soaking for some days in 1% $FeSO_4$, washing away the excess, and boiling. This theory fits the observation of Tinkler [36]) that black pigmentation could be induced in normal potatoes by contact with rusting iron during boiling. Cowie [8]) observed that blackening after cooking was confined to tubers grown on K-deficient plots which also had relatively high N-level. He suggested that potassium deficiency may bring about an increased concentration of iron in the tubers, in much the same way as it does in the nodal tissues of maize plants.

As has been stated above, a marked correlation was found by the author between the non-enzymatic discoloration of boiled potato tubers and the level of potassium supply. In potassium-deficient tubers this coloration was confined mainly to the stem end and to the surface layers just beneath the peel.

When cooked slices of both potassium-deficient and normal tubers were soaked in a one per cent $FeSO_4$ solution and exposed to the air, a deep bluish-green colour developed, particularly in the tissues outside the vascular ring, and at the stem end much more than at the bud end. This discoloration again was much more pronounced in the potassium-deficient tubers than in the normal ones.

Since the tissues which develop the non-enzymatic coloration are characterized by a very high content of *o*-dihydric phenol (see Table XVIII), it was suspected that the iron compound of the substance is responsible for this type of blackening. This was confirmed by the fact that when cooked non-blackening tuber slices were soaked first in catechol and then in $FeSO_4$ a deep blue-green coloration was produced over the whole surface. With dopa and $FeSO_4$ a similar colour

was formed. When a solution of catechol or dopa was given $Fe_2(SO_4)_3$ the pigment was formed instantaneously, but when $FeSO_4$ was used it was necessary to shake with air in order to develop the colour. Hence it is probable that the ferric compound of an *o*-dihydroxyphenol is responsible for the discoloration.

Since both the *o*-diphenol and the iron contents of the tubers will affect the degree of discoloration upon cooking, it is highly probable that under certain circumstances (high content of *o*-diphenol) a clear correlation between blackening and iron content will be found, while in other cases (low *o*-diphenol content) the effect of iron will be less clear.

Besides *o*-diphenol and iron contents a third factor helps to determine the degree of discoloration. This may be concluded from the results of the following experiments. After being boiled, slices of potatoes with an adequate K-nutrition were soaked in a solution of dopa or catechol and exposed to the air. No green-blue colour developed such as occurs in K-deficient tubers treated in the same manner. A similar result was obtained when the tubers were boiled in the *o*-diphenol solutions. Since soluble iron of the K-deficient tubers was not higher than that of tubers from plants supplied adequately

TABLE I*a*

Iron *) in Noordeling potato tubers (Exp. 589, 1948, analysed June, 1949).

Manuring		Half of tuber	Sol. Fe in dry matter p.p.m.	
kg K_2O per ha	kg N per ha	analysed	raw tissue	boiled tissue
0	120	bud	17.5	28.0
		stem	23.9	16.7
0	200	bud	17.6	35.8
		stem	18.4	30.9
100	120	bud	13.2	27.8
		stem	19.7	27.3
100	200	bud	13.9	25.8
		stem	18.4	25.4
600	120	bud	19.–	36.4
		stem	24.8	42.8
600	200	bud	17.0	36.3
		stem	22.8	37.6

*) 5 g of tuber tissue are ground in a porcelain mortar after addition of a knife point of ascorbic acid and 0.5 cc of glacial acetic acid to bring the pH to 4.4. The pulp is transferred to a 50 cc flask and made up to volume with a saturated solution of $(NH_4)_2SO_4$, in order to precipitate the protein. The latter is separated by filtration and the iron in the filtrate determined by adding a few cristals of *o*-phenanthroline and after 10 minutes' incubation at a temperature not below 20°C determining the resulting red colour in a colorimeter using a filter of 520 mμ [12] [9]).

with potassium (see Table I*a*), it must be assumed that in the former conditions were more favourable for the formation of the Fe-compound of *o*-dihydric phenols than in the latter.

As is stated on p. 61 Juul[12]) suggests that the degree of discoloration of cooked potatoes is closely related to the pH of the tissue. In the author's experiments, however, the difference in pH between normal and K-deficient tubers was so small (about 0.1) that it cannot have affected the degree of discoloration.

That the effect of the hydrogen ion concentration of the tissue is relatively small, was shown by boiling slices of potassium-deficient tubers in phosphate buffers of the following pH: 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.2 and 6.4. No differences in discoloration were found after exposure of the slices to the air. In a subsequent experiment phosphate buffers of pH 5.0, 5.4, 6.2 and 8.3, followed by a solution of $FeSO_4$ were added to pulped K-deficient tubers. No difference in degree of discoloration was observed between pH 5.0 and 5.4. At pH 6.2 the colour was somewhat deeper blue-green than at pH 5.4 and at pH 8.3 the pulp became black.

In the author's opinion the content of organic acids, particularly citric acid, is of much more importance than the pH in determining the degree of discoloration of K-deficient tubers. This is concluded from the following experiment. Slices of K-deficient tubers were boiled in a citrate buffer at pH 5.85. During boiling the tubers were pulped and the pulp was then exposed to the air. Discoloration was much less than in a pulp of the same pH treated with distilled water. When a solution of $FeSO_4$ had been added, the untreated pulp gave a much deeper colour than the pulp to which citrate was added. Apparently a great deal of the iron was bound by citric acid and could not react with the *o*-dihydric phenols. Succinic and malic acids gave a slight reduction, while glutamic and aspartic acids did not affect the degree of discoloration at all.

Since potassium-deficient tubers contain considerably less citric acid than those from plants with an adequate potassium supply (Juul), it is probable that owing to this the iron of K-deficient tubers will react with *o*-dihydric phenol more easily than it does when more citric acid is present.

Effect of the nitrogen, phosphate, and magnesium supplies on the discoloration after cooking. Tubers grown on plots differing in their N-,

P-, and Mg-dressings, and stored for about six months, were tested for discoloration after cooking in the manner described on p. 73. No clear effects of the magnesium and phosphate supplies were observed on the colour of the cooked potatoes. Nitrogen supply appeared to be of more importance. Nitrogen-deficient tubers after cooking were entirely white in colour as opposed to those amply dressed with nitrogen which became pale grey-green.

f. Attack on potassium-deficient potatoes by saprophytic micro-organisms. Potassium-deficient potatoes showing areas of black or grey-brown tissue are easily assailed by saprophytic micro-organisms. This accords well with the experiment described above using *Bact. prodigiosum*, in which it was seen that only those parts were attacked which in the corresponding uninoculated halves showed the black discolorations.

As the bruised tissue complexes are found at the stem end, the tubers are always attacked from that region (Plate IV). This phenomenon is very common when potassium-deficient tubers are stored. Often it can already be observed at the time of harvest. In the course of some months, however, the damage becomes much aggravated. Table II shows the percentage of tubers attacked by secondary micro-organisms.

TABLE II

Effect of potassium nutrition of potato plants on the attack by saprophytic micro-organisms on the tubers (December 1948)

kg K_2O per ha	Number of tubers tested	Percentage			
		Healthy	1/4 *)	2/4 *)	3/4 *)
0	1697	74.2	5.0	4.4	16.4
100	2142	91.9	3.2	1.7	3.1
400	1000	100.0	0.0	0.0	0.0

*) part of tuber affected.

It will be seen that potassium-deficient tubers are much more heavily attacked by saprophytic organisms than are those with a normal potassium supply.

4. *Tyrosine content of potato tubers grown under various conditions.* The potatoes used for the tyrosine analyses were grown on experimental fields in the years 1945–'48. These fields were laid out no

sandy or peat soils poor in one or more of the following nutritive elements: N, P, K, Mg and Cu. By supplying different amounts of these nutrients, potatoes were grown showing deficiency symptoms graded in severity.

An extensive report of the results from these field trials will be given elsewhere, but the yield data of those plots from which samples were taken for tyrosine determinations will be recorded here also. Only in this way is it possible to obtain an impression of the extent to which the crops suffered from shortage of mineral nutrients The main objection to the fertilizer experiments described by some investigators is that no yield data are recorded, so that it is impossible to assess the extent to which the plants in their experiments were suffering from inadequate supplies of mineral nutrients.

a. Effect of potassium and nitrogen supplies to potato plants on the tyrosine content of the tubers. Preliminary investigations concerning the effect of potassium and nitrogen dressings on the tyrosine content of the tubers were carried out in 1945.

Potatoes of the varieties Noordeling and Voran were grown on a slightly acid sandy soil containing about 10 per cent humus (exp. field 587). With no application of potassium salts, heavy symptoms of potassium deficiency occurred. When potassium sulphate was applied at the rate of 100 kg per ha, growth was much improved, but normal plants were obtained only when the salt was given at the rate of 800 kg per ha.

On August 9 five plants were harvested from each manurial treatment. Although by this time the plants had not ripened, considerable differences in yield were obtained as a result of the potassium and nitrogen treatments (Table III). The tubers were carefully harvested, taken to the laboratory and washed with tapwater. They were then used for chemical analysis. At this early date, black discolorations were found only in the samples grown without any potassium dressing. As usual the coloured patches occurred at the stem end of the tubers.

For the tyrosine determinations 50 g of tuber tissue were ground in a porcelain mortar; 20 cc of glacial acetic acid and 30 cc of water were added and the pulp separated on a Büchner funnel. The residue was washed with 2 per cent acetic acid and the filtrate made up to 200 cc. Aliquots were used for the tyrosine determination as described above. In these first determinations samples from coloured and white areas of the same potassium-deficient tubers were analysed separately. The results of these determinations are given in Table III.

TABLE III

Effect of potassium treatment and variety of potato on the free-tyrosine content of the tubers (Exp. 587, 1945)

Manuring		Yield	Variety	mg per 10 g of tissue		Per cent of dry matter	
kg K_2O /ha	kg N /ha	q tubers /ha		Soluble N	**Tyrosine**	Soluble N	**Tyrosine**
0	300	101.6	Noordeling *)	31.9	**5.5**	1.21	**0.21**
0	300		,, **)	23.2	**7.1**	0.76	**0.23**
0	300		,, †)	25.4	**4.2**	0.98	**0.16**
50	300	243.2	,, *)	23.–	**4.4**	0.91	**0.17**
400	300	320.–	,, *)	21.5	**1.2**	0.89	**0.05**
0	100	107.2	Voran *)	24.9	**3.–**	1.18	**0.14**
0	300	131.2	,, *)	26.9	**3.–**	1.09	**0.12**
400	100	279.2	,, *)	15.1	**1.8**	0.76	**0.09**
400	300	340.8	,, *)	20.1	**1.5**	0.93	**0.07**

*) average tissue sample.

**) sample from discoloured areas.

†) sample of white tissue from discoloured tubers.

These data show that potassium-deficient tubers have a considerably higher tyrosine content than those with a normal potassium supply. Parts of the tuber which are liable to blackening are extremely rich in tyrosine. The variety Noordeling, which is very prone to blacken, contains almost twice as much as Voran which is much less so.

The results obtained in this preliminary investigation were confirmed by those from a great many similar experiments on different soils and in four successive years.

Tests of 1946. (Effect of storage on the tyrosine content of potassium-deficient tubers).

In 1946 samples were investigated from experimental field 589, located on a sandy soil with a pH of about 5 and containing approximately 10 per cent organic matter. This field was likewise very poor in available potassium as may be seen from the yield data given in Table IV. Potassium and nitrogen were supplied in different amounts, and the whole field was treated amply with phosphatic and magnesium fertilizers.

The first analysis of tubers took place on July 22 when 10 plants from every manurial treatment were harvested. At that date the plants without any potassium dressing showed heavy symptoms of K-deficiency on the leaves. Those supplied with potassium sulphate at the rate of 100 kg K_2O per ha displayed light deficiency symptoms. Bluish-black discolorations occurred only at the stem end of the tubers which had received no potassium.

Besides tyrosine, a number of other nitrogenous compounds were determined. Of these the values for protein nitrogen and total soluble nitrogen are recorded in the following tables.

TABLE IV

Effect of potassium and nitrogen treatments *) on yield of tubers and protein nitrogen, soluble non-protein nitrogen and free-tyrosine contents of potatoes (Exp. 589, 1946)

Variety and date of analysis	Manuring		Yield	mg per 10 g of tuber tissue			% of dry matter			Tyrosine-N as % of sol. non-protein-N
	kg K_2O per ha	kg N /ha	q †) tubers /ha	Protein-N	Soluble non-protein-N	**Tyrosine** °)	Protein-N	Soluble non-protein-N	**Tyrosine**	
Noordeling July 22	0	0	72.8	21.7	17.8	**7.5**	0.93	0.76	**0.32**	**3.27**
	0	100	75.2	25.1	22.8	**7.1**	1.07	0.97	**0.30**	**2.40**
	0	300	84.5	24.6	24.5	**6.3**	1.06	1.06	**0.27**	**1.97**
	50	0	95.6	16.5	8.6	**3.3**	0.69	0.36	**0.14**	**3.-**
	50	100	187.2	19.5	18.1	**5.6**	0.88	0.82	**0.25**	**2.35**
	50	300	151.5	18.6	19.–	**4.-**	0.87	0.88	**0.18**	**1.58**
	300	0	146.4	17.3	9.5	**2.7**	0.84	0.46	**0.13**	**2.18**
	300	100	193.2	21.–	14.2	**2.7**	0.97	0.66	**0.12**	**1.41**
	300	300	180.5	22.5	18.3	**2.5**	1.07	0.87	**0.12**	**1.07**
Voran July 22	0	100	60.8	23.2	20.8	**5.4**	1.11	0.99	**0.26**	**2.03**
	50	100	168.4	16.4	14.7	**3.6**	0.90	0.80	**0.20**	**1.94**
	50	300	142.9	20.1	15.4	**2.7**	1.05	0.80	**0.14**	**1.36**
	300	0	130.–	11.2	4.8	**1.3**	0.63	0.27	**0.07**	**2.12**
	300	100	144.8	16.6	10.4	**1.5**	0.93	0.58	**0.08**	**1.12**
Noordeling October 10	0	0	67.3	24.9	20.4	**7.7**	1.11	0.91	**0.34**	**2.93**
	0	100	76.1	24.6	24.4	**7.-**	1.07	1.07	**0.31**	**2.22**
	0	300	91.8	—	24.5	**6.8**	—	1.02	**0.28**	**2.13**
	50	0	152.1	21.3	13.6	**4.1**	0.82	0.52	**0.16**	**2.33**
	50	100	202.4	24.–	17.2	**5.5**	0.95	0.68	**0.22**	**2.48**
	50	300	209.4	28.2	24.4	**5.1**	1.19	1.03	**0.21**	**1.60**
	300	0	169.8	21.8	9.1	**3.-**	0.91	0.38	**0.12**	**2.54**
	300	100	266.3	23.9	15.5	**3.3**	0.98	0.64	**0.14**	**1.64**
	300	300	327.5	—	18.9	**3.1**	—	0.74	**0.12**	**1.25**
Voran October 10	0	0	64.1	21.1	19.7	**5.5**	1.09	1.01	**0.28**	**2.15**
	0	300	79.–	24.5	21.6	**4.3**	1.16	1.02	**0.20**	**1.52**
	300	0	261.–	12.–	6.–	**1.8**	0.55	0.28	**0.08**	**2.20**
	300	300	460.8	20.5	16.6	**3.-**	0.91	0.74	**0.13**	**1.36**
Noordeling March 24, 1947	0	0	— §)	28.5	20.6	**9.2**	1.12	0.80	**0.36**	**3.46**
	0	100	—	29.1	27.3	**8.1**	1.24	1.16	**0.34**	**2.27**
	0	300	—	28.1	28.7	**7.1**	1.08	1.10	**0.27**	**1.89**
	50	0	—	23.9	16.–	**4.2**	0.84	0.56	**0.15**	**2.07**
	50	100	—	29.6	24.6	**7.2**	1.08	0.89	**0.26**	**2.26**
	50	300	—	34.6	29.5	**6.8**	1.12	0.98	**0.23**	**1.82**
	300	0	—	23.1	14.5	**3.3**	0.92	0.58	**0.13**	**1.73**
	300	100	—	28.6	18.6	**3.5**	1.10	0.71	**0.14**	**1.52**
	300	300	—	31.–	28.–	**3.6**	1.04	0.94	**0.12**	**0.99**
Voran March 24, 1947	0	0	—	23.2	21.5	**6.2**	1.03	0.95	**0.28**	**2.28**
	0	300	—	28.3	28.–	**4.7**	1.25	1.23	**0.21**	**1.32**
	300	0	—	14.2	9.9	**1.8**	0.59	0.42	**0.08**	**1.41**
	300	300	—	27.8	20.9	**3.-**	1.06	0.81	**0.12**	**1.11**

*) K was supplied as K_2SO_4, N as NH_4NO_3 (amm. nitrate limestone).
§) Same as October 10.
†) 1 q = 100 kg
°) acetic-acid-treated samples.

The data from the first harvest are given in Table IV. They reveal a close relation between the potassium supply to the plants and the free-tyrosine content of the tubers. Although total soluble non-protein nitrogen was high in potassium-deficient tubers, the relative content of tyrosine nitrogen was considerably higher there than in tubers with a normal potassium supply.

It is remarkable that with improving nitrogen supply the tyrosine content increased only insignificantly. The highest dressing of nitrogen even gave lower values. Since the total soluble nitrogen increased very markedly, mainly as a result of a rise in asparagine and glutamine, the relative tyrosine-N values show a considerable drop with increasing nitrogen supply.

The variety Voran, which is considerably less liable to discolour, had a consistently lower tyrosine content than Noordeling.

The second analysis took place some days after harvesting the crop from the whole field, on October 10, 11 and 12. The potatoes were harvested and carried very carefully, so that discoloration occurred only to a small extent in the potassium-deficient tubers. These tubers blackened very easily, however, as appeared when they were shaken for five minutes in a flask and then devided longitudinally. Great areas of tissue at the stem end turned reddish and then bluish-black as described above.

The same was true, though to a somewhat less degree, of the tubers dressed with 50 kg of K_2O per ha. In those supplied with 300 kg of K_2O light discolorations only were seen.

A third analysis was carried out on March 24 and 25 when the tubers had been stored for about five months at approximately 10°C.

The results of the second and third analyses in general agree well with those of the first harvest. Although the tyrosine values, calculated on tuber tissue, increased considerably during storage, this rise was largely due to loss of water from the stored tubers. On a dry matter basis the increments were comparatively small; and when calculated in terms of total soluble nitrogen, tyrosine nitrogen even showed a fall. This is due to the fact that other soluble non-protein compounds increased to a greater extent than tyrosine.

Tests of 1947. (Effect of potassium and nitrogen dressings on the tyrosine content of stem and bud ends of potatoes).

In 1947, samples were available from experimental field 650, located on a reclaimed peat soil with 10 per cent organic matter. Potassic and nitrogenous fertilizers were supplied at different rates.

TABLE V

Effect of potassium and nitrogen dressings *) on yield of tubers and protein nitrogen, soluble non-protein nitrogen and free-tyrosine contents of Noordeling potatoes (Exp. 650, 1947)

Manuring		Yield q tubers /ha (October)	Half of tuber analysed	mg per 10 g of tuber tissue				% of dry tuber tissue				**Tyrosine-N (Na_2SO_3) as% of soluble non-protein N**
				Protein-N	Soluble non-protein-N	**Tyrosine**		Protein-N	Soluble non protein-N	**Tyrosine**		
kg K_2O /ha	kg N /ha					Na_2SO_3	Acet. Acid			Na_2SO_3	Acet. Acid	
0	60	180.4	bud	26.6	23.5	**6.9**	**6.2**	1.22	1.08	**0.32**	**0.29**	**2.30**
			stem	30.–	27.8	**7.2**	**6.7**	1.19	1.11	**0.29**	**0.27**	**2.02**
100	60	265.6	bud	26.2	15.9	**2.9**	**2.6**	0.94	0.57	**0.10**	**0.09**	**1.36**
			stem	26.3	20.3	**4.1**	**3.5**	0.85	0.65	**0.13**	**0.11**	**1.59**
300	60	315.6	bud	24.1	11.8	**1.6**	**1.8**	0.96	0.47	**0.06**	**0.07**	**1.05**
			stem	27.–	15.4	**2.6**	**2.4**	0.93	0.53	**0.09**	**0.08**	**1.31**
600	0	276.8	bud	22.5	11.2	**1.6**	**1.5**	0.98	0.49	**0.07**	**0.07**	**1.10**
			stem	20.6	11.9	**2.1**	**2.3**	0.86	0.50	**0.09**	**0.10**	**1.39**
600	60	314.4	bud	27.2	12.9	**1.4**	**1.2**	1.06	0.50	**0.06**	**0.05**	**0.87**
			stem	26.–	17.3	**2.3**	**2.1**	1.04	0.69	**0.09**	**0.08**	**1.01**
600	200	323.5	bud	35.1	24.3	**2.1**	**1.6**	1.26	0.87	**0.08**	**0.06**	**0.67**
			stem	36.8	31.–	**2.8**	**2.8**	1.23	1.04	**0.09**	**0.09**	**0.67**

*) K was supplied as K_2SO_4, N as NH_4NO_3 (ammonium nitrate limestone). Superphosphate was supplied as a basic dressing at the rate of 120 kg P_2O_5 per ha.

The potatoes were harvested on October 16 and 17 when the plants in all treatments had ripened. Analyses were made on samples harvested in the first week of September, top and stem halves of the tubers being analysed separately. A further comparison was made of the relative effects of treatment with acetic acid and sodium sulphite when these are used as inhibitors of the oxidation of tyrosine (see analytical methods).

The yield data and the values for protein and soluble non-protein nitrogen and those for tyrosine are shown in Table V. As in the previous experiments, a close correlation was found between the potassium supply to the tubers and their tyrosine content. Potassium-deficient tubers had a content nearly five times as high as that of tubers supplied amply with potassium.

Stem ends of the tubers had considerably higher tyrosine values than the bud ends. This was not the case, however, in those tubers grown with no potassium dressing and which showed great areas of discoloured tissue at their stem ends. Apparently a substantial part of the tyrosine in this tissue had been converted into black oxidation products.

Samples treated with acetic acid showed somewhat lower tyrosine values than those treated with sodium sulphite. Apparently slight oxidation had still occurred in the acetic-acid-treated samples.

Similar results to the above were obtained using tubers grown on some other fields in different years (see Tables VI and XII).

TABLE VI

Effect of potassium treatment on the free-tyrosine content of Noordeling potatoes (Exp. 589, 1948, analysed January 17, 1949)

Kg K_2O *) per ha	Yield q tubers per ha	mg per 10 g of tuber tissue			% of dry tuber tissue			**Tyrosine-N** as % of soluble non-protein-N
		Protein-N	Soluble non-protein-N	**Tyrosine** †)	Protein-N	Soluble non-protein-N	**Tyrosine**	
0	169	29.4	29.2	**6.3**	1.27	1.26	**0.27**	**1.66**
100	349	24.9	27.4	**5.6**	0.99	1.09	**0.22**	**1.56**
600	384	23.1	22.8	**3.6**	1.04	1.03	**0.16**	**1.20**
1000	359	21.8	21.6	**3.3**	1.—	0.99	**0.15**	**1.17**

*) K was supplied as K_2SO_4, N at the rate of 200 kg per ha as ammonium nitrate limestone, P at the rate of 100 kg P_2O_5 per ha as double superphosphate.

†) Na_2SO_3-treated samples.

Effect of potassium supply on the tyrosine content of different potato varieties. It is a well-known fact that different potato varieties are not equally liable

to discolour when inadequately supplied with potassium. This was shown clearly in the experiments described above in which usually two varieties were grown. Noordeling, which is very liable to blacken has a substantially higher tyrosine content than Voran, which is much less so.

In 1948 several varieties were grown under various conditions of potassium supply on a soil initially poor in potassium (field trial 1031). Yield data, extent of blackening and values for protein nitrogen, soluble non-protein nitrogen and free tyrosine are shown in Table VII).

It will be seen that of the varieties tested, Noordeling was by far the most prone to blacken. Its tyrosine content was also the highest of all varieties investigated. In addition, the cells of this variety are very readily injured. Bevelander, which was likewise high in tyrosine, showed relatively little tendency to blacken, apparently because its cells are much less easily injured. Wilpo possessed opposite characteristics to those of Bevelander: a moderate content of tyrosine but tissues which are readily damaged.

Isolation of tyrosine. For the isolation of tyrosine, according to the method described on p. 63 samples of both K-deficient and normal Noordeling tubers, grown on Exp. field 589 (1946), were used. Considerable amounts of pure tyrosine have been isolated from K-deficient tubers. The crystals obtained showed the typical needles of tyrosine, while the C, H and N contents found were close to those of the pure substance.

Found: C: 62.5%, H: 6.9%, N: 7.6%, Tyrosine: C: 59.6, H: 6.1, N: 7.7).

No tyrosine could be isolated from tubers amply supplied with potassium.

b. Effect of phosphorus supply on the tyrosine content of potatoes.

Fertilizer trials with different amounts of phosphate were carried out in two consecutive years on slightly acid sandy soils containing about 5 per cent humus and poor in available phosphate. As in the experiments with potassium, usually two varieties of potato were grown, viz. Noordeling and Voran. Different combinations of nitrogen and phosphate treatment were applied. The tubers were treated and analysed in the same way as in the potassium tests. Yield data from these experiments will be recorded only

TABLE

Effect of potassium and nitrogen treatments on yield, extent of blackening of raw tubers, potato.

Variety	Manuring *)		Yield q tubers per ha	mg per 10 g of tuber tissue		
	kg K_2O per ha	kg N per ha		Protein-N	Soluble non-protein-N	Tyrosine §)
Noordeling . . .	0	100	174	26.6	23.3	**6.7**
	0	140	153	27.–	23.9	**7.–**
	500	140	338	24.1	19.9	**3.–**
Voran	0	100	187	20.7	20.2	**5.–**
	0	140	182	20.3	22.–	**4.1**
	500	140	437	20.7	17.1	**2.1**
Rode Star . . .	0	100	169	27.1	23.2	**6.4**
	0	140	159	25.8	25.9	**6.8**
	500	140	356	18.8	15.8	**3.2**
Gloria	0	100	205	19.6	21.5	**4.2**
	0	140	168	22.7	26.4	**5.1**
	500	140	454	16.5	13.4	**2.4**
Libertas . . .	0	100	124	23.4	26.–	**5.2**
	0	140	132	23.–	28.1	**5.4**
	500	140	363	19.2	15.3	**2.4**
Eigenheimer . .	0	100	194	23.4	24.8	**2.7**
	0	140	191	24.6	29.7	**3.1**
	500	140	438	19.1	17.3	**2.3**
Bintje	0	100	161	20.2	29.–	**4.9**
	0	140	227	20.9	30.–	**3.9**
	500	140	423	13.9	17.4	**2.–**
Bevelander . . .	0	100	151	21.3	31.6	**6.6**
	0	140	214	24.5	31.2	**6.8**
	500	140	331	19.7	19.4	**3.1**
Eersteling. . . .	0	100	176	14.3	26.7	**4.8**
	0	140	158	17.9	30.7	**5.6**
	500	140	202	15.8	21.–	**3.1**
Industrie	0	100	168	22.7	22.7	**5.3**
	0	140	170	22.2	22.8	**5.2**
	500	140	373	19.7	16.8	**2.8**
Wilpo	0	100	190	22.5	22.9	**4.2**
	0	140	208	19.8	23.2	**4.4**
	500	140	335	17.7	15.2	**3.–**
Record	0	100	151	19.4	27.4	**3.9**
	0	140	141	19.6	30.2	**5.3**
	500	140	350	19.1	14.5	**2.3**

*) K was supplied as K_2SO_4, N as ammonium nitrate limestone, P as a basic dressing at the rate of 100 kg P_2O_5 per ha as double superphosphate and $MgSO_4$ at the rate of 30 kg MgO per ha.

†) 1 = 10% of total tissue discoloured, 2 = 20% etc.

§) Na_2SO_3-treated samples.

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protein nitrogen, soluble non-protein nitrogen, and free-tyrosine contents of several varieties of Exp. 1031, 1948)

As % of dry tissue tuber			**Tyrosine**-N as % of soluble non-protein-N	Extent of blackening †)	
Protein-N	Soluble non-protein-N	**Tyrosine**		upon 5 minutes shaking October '48	after 6 months storage March '49
1.14	1.—	**0.29**	**2.22**	6	3
1.18	1.05	**0.30**	**2.25**	5	2
1.02	0.85	**0.13**	**1.17**	1.5	0
0.94	0.92	**0.23**	**1.92**	2	1.5
0.89	0.97	**0.18**	**1.45**	1	0
0.99	0.82	**0.10**	**0.93**	0.5	0
1.04	0.89	**0.24**	**2.12**	3	1.5
1.03	1.04	**0.27**	**2.03**	2	1.–
0.77	0.65	**0.13**	**1.63**	1	0
0.89	0.97	**0.19**	**1.50**	2.5	0.5
1.05	1.22	**0.24**	**1.49**	2.5	0.5
0.80	0.65	**0.12**	**1.39**	1.5	0
1.14	1.27	**0.25**	**1.55**	2	0.5
0.97	1.18	**0.23**	**1.50**	2	0
0.68	0.54	**0.09**	**1.23**	2	0
1.05	1.11	**0.12**	**0.85**	1	0.5
1.08	1.31	**0.14**	**0.81**	2.5	0.5
0.84	0.76	**0.10**	**1.02**	0.5	0
0.98	1.40	**0.24**	**1.31**	0.5	0
1.02	1.47	**0.19**	**1.–**	0.5	0
0.65	0.82	**0.10**	**0.90**	0	0
0.89	1.32	**0.27**	**1.60**	1.5	0
0.95	1.21	**0.26**	**1.68**	1.5	0
0.86	0.85	**0.13**	**1.23**	1.5	0
0.76	1.42	**0.25**	**1.39**	1	0
0.89	1.53	**0.28**	**1.41**	1	0
0.85	1.12	**0.17**	**1.14**	0.5	0
1.08	1.08	**0.25**	**1.78**	1.5	2
0.96	0.99	**0.22**	**1.74**	1	1
0.75	0.64	**0.10**	**1.27**	1.5	0.5
1.03	1.05	**0.19**	**1.42**	4	3
0.97	1.14	**0.22**	**1.47**	3	1.5
0.80	0.69	**0.13**	**1.51**	1.5	0
0.97	1.36	**0.19**	**1.10**	2	1
0.93	1.43	**0.25**	**1.37**	3	0.5
0.85	0.64	**0.10**	**1.20**	1	0

for those samples in which tyrosine was estimated. Full details will be published elsewhere. Table VIII contains the results of experiment 907, Table IX those of experiment 965. In both cases a large response to phosphorus was obtained as can be seen from the yield data.

As shown in Table VIII, in experiment 907 the tyrosine values were low and practically unaffected by the phosphate supply to the

TABLE VIII

Effect of phosphorus and nitrogen treatments on yields of tubers and protein nitrogen, soluble non-protein nitrogen, and free-tyrosine contents of potatoes (Exp. 907, 1946)

Variety and date of analysis	Manuring *)		Yield q tubers /ha	mg per 10 g of tuber tissue			% of dry matter			Tyrosine-N as % of soluble non-protein-N
	kg P_2O_5 /ha	kg N /ha		Protein-N	Soluble non-protein-N	Tyrosine§)	Protein-N	Soluble non-protein-N	Tyrosine	
Noordeling July 24	0	0	88.–	17.2	8.–	**2.1**	0.77	0.39	**0.09**	**1.78**
	0	100	73.5	21.9	15.3	**1.9**	1.18	0.83	**0.10**	**0.93**
	0	300	65.4	23.8	17.5	**2.3**	1.23	0.91	**0.12**	**1.02**
	100	0	87.7	17.8	8.4	**2.–**	0.76	0.36	**0.09**	**1.93**
	100	100	123.3	19.8	11.6	**2.3**	0.85	0.50	**0.10**	**1.55**
	100	300	87.9	20.9	17.5	**2.1**	1.07	0.89	**0.11**	**0.96**
	500	0	161.9	18.4	9.4	**2.1**	0.85	0.43	**0.10**	**1.80**
	500	100	159.3	20.5	12.9	**2.3**	1.04	0.65	**0.12**	**1.43**
	500	100 †)	173.7	21.1	13.8	**2.8**	1.11	0.73	**0.15**	**1.59**
	500	300	170.8	22.5	16.3	**2.5**	1.14	0.83	**0.13**	**1.21**
	500	300 †)	151.6	23.5	19.6	**3.1**	1.14	0.95	**0.15**	**1.22**
Voran July 24	0	300 †)	48.–	21.1	15.1	**2.3**	1.29	0.92	**0.14**	**1.18**
	100	300 †)	107.4	20.9	15.3	**1.7**	1.19	0.87	**0.10**	**0.89**
	500	300 †)	224.9	20.9	17.3	**1.9**	1.20	0.99	**0.11**	**0.86**
Noordeling October 14	0	0	150.–	21.5	9.2	**2.2**	0.89	0.38	**0.09**	**1.87**
	0	100	212.8	26.9	15.4	**2.2**	1.02	0.58	**0.08**	**1.12**
	0	300	209.5	29.9	20.6	**3.7**	1.22	0.84	**0.15**	**1.40**
	0	300 †)	204.4	31.–	26.3	**3.5**	1.25	1.06	**0.14**	**1.03**
	100	300	241.5	28.–	20.3	**3.1**	1.16	0.84	**0.13**	**1.19**
	500	0	201.4	19.8	8.9	**2.7**	0.80	0.36	**0.11**	**2.36**
	500	100	279.8	24.2	14.8	**2.5**	1.04	0.64	**0.11**	**1.29**
	500	300	294.3	28.9	18.9	**2.7**	1.19	0.78	**0.11**	**1.10**
	500	300 †)	291.8	27.8	20.6	**3.–**	1.14	0.84	**0.12**	**1.14**
Voran October 14	0	300	305.3	24.–	18.5	**3.3**	1.07	0.82	**0.15**	**1.42**
	500	0	288.–	13.4	7.7	**2.–**	0.63	0.36	**0.09**	**2.02**
	500	300	452.3	20.8	15.4	**2.–**	0.97	0.71	**0.09**	**1.02**
Noordeling March 26, 1947	0	0	—	23.9	13.1	**1.9**	0.93	0.51	**0.07**	**1.06**
	0	100	—	24.2	21.7	**2.5**	0.92	0.83	**0.10**	**0.93**
	0	300	—	33.2	24.7	**2.9**	1.25	0.92	**0.11**	**0.93**
	100	300	—	30.8	22.6	**2.7**	1.21	0.90	**0.10**	**0.86**
	500	0	—	22.–	10.9	**2.1**	0.82	0.40	**0.08**	**1.54**
	500	100	—	27.3	17.1	**2.4**	1.03	0.64	**0.09**	**1.09**
	500	300	—	32.1	23.1	**2.7**	1.18	0.85	**0.10**	**0.91**
	500	300 †)	—	30.2	28.9	**4.2**	1.14	1.09	**0.16**	**1.13**
Voran March 26, 1947	0	300	—	28.6	22.1	**2.9**	1.24	0.96	**0.13**	**1.05**
	500	0	—	13.6	10.3	**2.–**	0.58	0.44	**0.08**	**1.46**
	500	300	—	23.2	19.–	**2.2**	1.–	0.82	**0.10**	**0.90**

*) P was supplied as superphosphate, N as $Ca(NO_3)_2$. K_2SO_4 at the rate of 300 kg K_2O per ha and $MgSO_4$ at 100 kg MgO per ha were supplied as basic dressings.

§) acetic-acid-treated samples.

†) N supplied as $(NH_4)_2SO_4$.

plants. Storage for five months resulted in a small drop in tyrosine content when calculated on a dry matter basis. The effect of increased nitrogen supply on the tyrosine values was likewise very small.

The results of experiment 965 (1947) are in full agreement with those of the preceding year (Table IX).

TABLE IX

Effect of phosphorus and nitrogen dressings *) on yield of tubers and protein nitrogen, soluble non-protein nitrogen and free-tyrosine contents of potatoes

Variety and date of analysis	Manuring		Yield q tubers ha	mg per 10 g of tuber tissue			% of dry matter			Tyrosine-N as % of soluble non-protein-N
	kg P_2O_5 /ha	kg N /ha		Protein-N	Soluble non-protein-N	Tyrosine †)	Protein-N	Soluble non-protein-N	Tyrosine	
Noordeling December 12	0	0	106.7	24.–	17.1	**1.1**	1.08	§ 0.76	**0.05**	**0.51**
	0	100	134.3	26.9	19.4	**1.5**	1.08	0.78	**0.06**	**0.60**
	0	300	120.9	29.8	23.8	**1.–**	1.27	1.01	**0.04**	**0.34**
	300	0	121.8	22.3	12.4	**1.3**	0.93	0.52	**0.05**	**0.81**
	300	100	247.2	28.–	16.9	**1.8**	1.06	0.64	**0.07**	**0.84**
	300	300	230.5	28.7	24.4	**1.7**	1.23	1.04	**0.07**	**0.53**
Voran December 12	0	300	138.5	17.8	19.5	**1.2**	1.01	1.10	**0.07**	**0.49**
	300	300	321.6	22.–	20.8	**1.7**	1.09	1.03	**0.09**	**0.68**

*) P was supplied as double superphosphate, N as $Ca(NO_3)_2$. K_2SO_4 at the rate of 300 kg K_2O per ha and $MgSO_4$ at 100 kg MgO/ha were supplied as basic dressings.

†) Na_2SO_3-treated samples.

The tyrosine values were again very low and practically unaffected either by phosphate or nitrogen treatments.

No discolorations were seen in the tubers from these experiments.

c. Effect of magnesium supply on the tyrosine content of potatoes.

Samples of magnesium-deficient potatoes were obtained from experimental field 662, located on a slightly acid sandy soil containing about 5 per cent organic matter. This field was poor in available magnesium, and cereals as well as potatoes responded clearly to magnesium dressings (Table X). Harvesting and analysis of the potatoes were carried out in the same manner as in the other fertilizer experiments.

TABLE X

Effect of magnesium and nitrogen treatments on yield of tubers and protein nitrogen, soluble non-protein nitrogen, and free-tyrosine contents *) of potatoes (Exp. 662, 1946)

Variety and date of analysis	Manuring †)		Yield q tubers /ha	mg per 10 g of tuber tissue			% of dry matter			Tyrosine-N as % of soluble non-protein N
	kg $MgSO_4$ /ha	kg N /ha		Protein N	Soluble non-protein-N	**Tyrosine**	Protein N	Soluble non-protein-N	**Tyrosine**	
Noordeling	0	150	130.1	23.2	19.2	**3.6**	1.06	0.88	**0.16**	**1.41**
July 26	0	300	115.6	25.8	20.1	**3.1**	1.15	0.90	**0.14**	**1.20**
	200	150	159.2	22.8	17.2	**3.1**	1.05	0.79	**0.14**	**1.37**
	200	300	153.2	23.5	20.6	**3.4**	1.10	0.96	**0.16**	**1.29**
Voran	0	150	154.–	18.7	12.2	**1.9**	0.94	0.62	**0.10**	**1.25**
July 26	0	300	142.–	22.4	16.4	**2.5**	1.15	0.84	**0.13**	**1.20**
	200	150	237.1	17.4	10.6	**1.4**	0.90	0.55	**0.07**	**0.98**
	200	300	151.6	20.–	14.5	**2.1**	1.03	0.75	**0.11**	**1.13**
Noordeling	0	0	90.–	20.5	9.6	**2.2**	0.81	0.38	**0.09**	**1.77**
	0	100	174.–	23.4	15.5	**3.5**	0.97	0.64	**0.14**	**1.75**
October 16	0	300	141.–	29.6	26.5	**4.–**	1.20	1.07	**0.16**	**1.18**
	200	0	92.–	19.8	9.7	**1.5**	0.77	0.38	**0.06**	**1.23**
	200	100	246.–	22.–	10.5	**2.3**	0.87	0.41	**0.09**	**1.72**
	200	300	313.–	28.5	21.7	**3.6**	1.11	0.85	**0.14**	**1.27**
Voran	0	300	258.–	21.3	18.5	**2.4**	0.94	0.82	**0.11**	**1.04**
October 16	200	300	442.–	20.3	16.9	**2.4**	0.92	0.76	**0.11**	**1.12**
Noordeling	0	0	—	24.7	12.6	**2.2**	0.87	0.44	**0.08**	**1.23**
March 27,	0	100	—	27.8	20.4	**3.6**	1.14	0.84	**0.15**	**1.38**
1947	0	300	—	33.8	32.6	**4.–**	1.24	1.20	**0.15**	**0.93**
	200	0	—	22.–	12.4	**1.9**	0.82	0.46	**0.07**	**1.18**
	200	100	—	27.7	18.–	**2.9**	1.01	0.65	**0.10**	**1.24**
	200	300	—	38.4	30.8	**3.5**	1.23	0.98	**0.11**	**0.87**
Voran	0	300	—	30.8	25.8	**3.4**	1.15	0.96	**0.13**	**1.05**
March 27, 1947	200	300	—	27.3	23.2	**3.1**	1.04	0.88	**0.12**	**1.06**

*) Acetic-acid-treated samples.

†) N supplied as $(NH_4)_2SO_4$. Double superphosphate at the rate of 100 kg P_2O_5 per ha and K_2SO_4 at 300 kg K_2O per ha were supplied as a basic dressing.

The results of the 1946 tests show that the magnesium-deficient potatoes had somewhat higher tyrosine values than those with a normal magnesium supply (Table X). As nitrogen nutrition improved tyrosine increased rather considerably.

The data of the 1947 tests (Table XI) agree with those of the preceding year in so far as the effect of magnesium is concerned.

TABLE XI

Effect of magnesium and nitrogen dressings *) on yield of tubers, and protein nitrogen, soluble non-protein nitrogen and free-tyrosine contents of potatoes (Exp. 662, 1947)

Variety and date of analysis	Manuring		Yield q tuber /ha	mg per 10 g of tuber tissue			% of dry matter			Tyrosine-N as % of soluble non-protein-N
	kg $MgSO_4$ /ha	kg N /ha †)		Protein-N	Soluble non-protein-N	**Tyrosine** °)	Protein-N	Soluble non-protein-N	**Tyrosine**	
Noordeling December 12	0	0	132.1	24.3	16.7	**2.5**	1.10	0.75	**0.11**	**1.15**
	0	150	234.2	27.3	18.2	**3.1**	1.12	0.75	**0.13**	**1.33**
	0	300	141.8	29.4	24.8	**2.4**	1.12	0.95	**0.09**	**0.76**
	0	300§)	199.4	29.7	28.9	**3.-**	1.19	1.16	**0.12**	**0.80**
	200	0	152.4	19.9	10.5	**2.2**	0.90	0.47	**0.10**	**1.60**
	200	150	286.7	28.3	15.1	**2.2**	1.07	0.57	**0.09**	**1.15**
	200	300	249.4	32.5	22.2	**1.9**	1.25	0.86	**0.07**	**0.66**
	200	300§)	244.2	32.8	30.2	**1.9**	1.28	1.17	**0.07**	**0.49**
Voran	0	0	184.4	12.6	10.7	**1.7**	0.73	0.62	**0.10**	**1.20**
	0	300	278.8	20.6	22.9	**1.9**	0.88	0.97	**0.08**	**0.66**
	200	0	194.3	11.2	10.7	**1.8**	0.57	0.54	**0.09**	**1.29**
	200	300	416.9	22.1	22.7	**1.8**	0.93	0.96	**0.08**	**0.61**

*) Basic dressing : double superphosphate at the rate of 100 kg P_2O_5 per ha and K_2SO_4 at 300 kg K_2O per ha.
†) N supplied as $Ca(NO_3)_2$.
§) N supplied as $(NH_4)_2SO_4$.
°) Na_2SO_3-treated samples.

In contrast to the experiment of 1946 tyrosine did not increase with the nitrogen supply.

In both years Noordeling contained considerably more tyrosine than Voran.

In accordance with the tyrosine contents the magnesium-deficient tubers were somewhat more prone to discolour after rough treatment than were those with a normal magnesium supply. Compared with potassium-deficient tubers, however, the blackening was only slight.

d. Effect of copper supply on the tyrosine content of potato tubers. As stated above, potato tubers grown on a soil poor in copper as well as in potassium are

considerably less liable to show discolorations than those grown on the same soil treated with copper sulphate.

This was observed for the first time in 1947 on a newly reclaimed heath soil poor in copper (exp. 966). The results obtained in 1948 (exp. 1011) were more convincing, however, and therefore will be described in some detail.

Experimental field 1011 was likewise located on a newly reclaimed heath soil dressed with phosphate and magnesium sulphate and supplied with calcium carbonate to bring the pH to about 5.0. Available copper, as determined by the use of *Aspergillus niger* *), was found to be very low. Potassium dressings were applied as K_2SO_4 at rates equivalent to 0, 25, 50, 100, 200 and 400 kg of K_2O per ha, each dressing being combined with three levels of copper supply (0, 10 and 100 kg of $CuSO_4.5H_2O$ per ha).

It is a well-known fact that potatoes constitute one of those crops which are very insensitive to copper deficiency (see [18]). Nevertheless

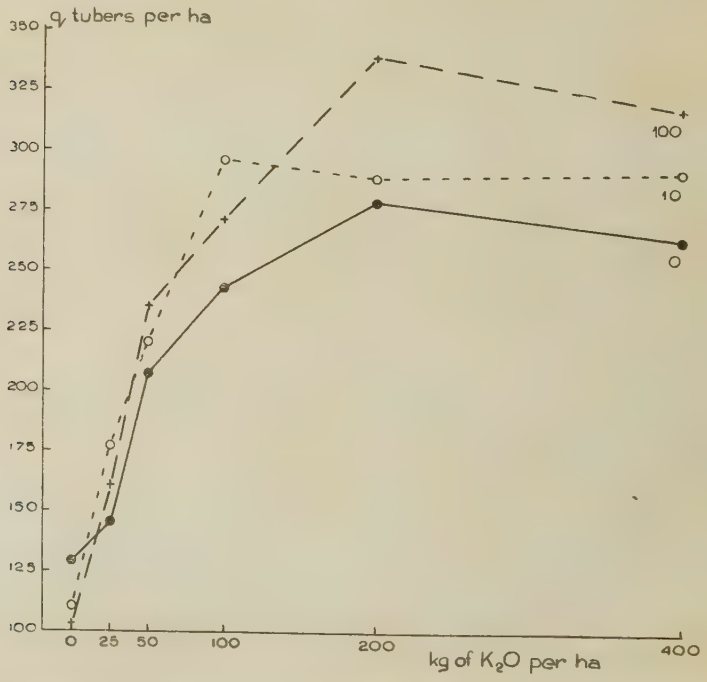
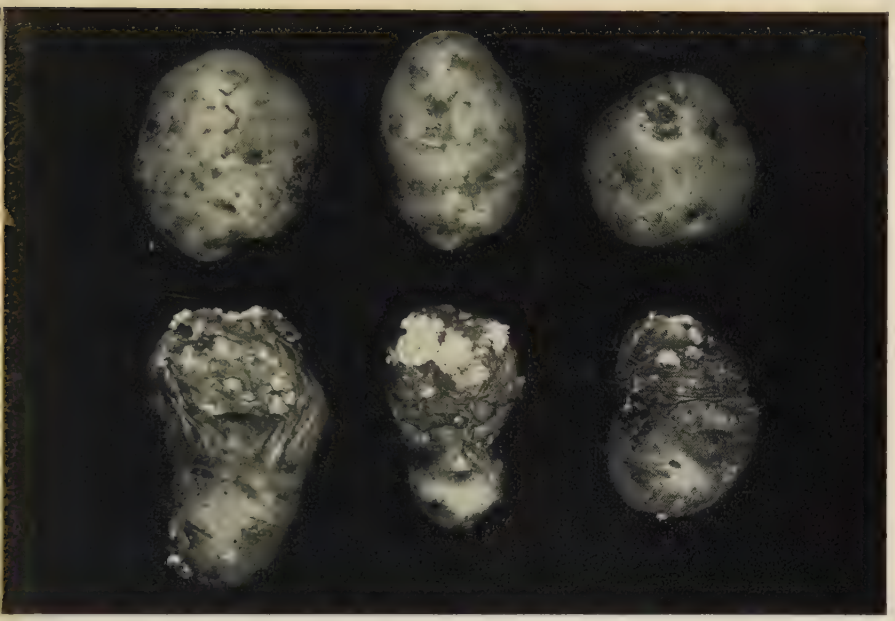
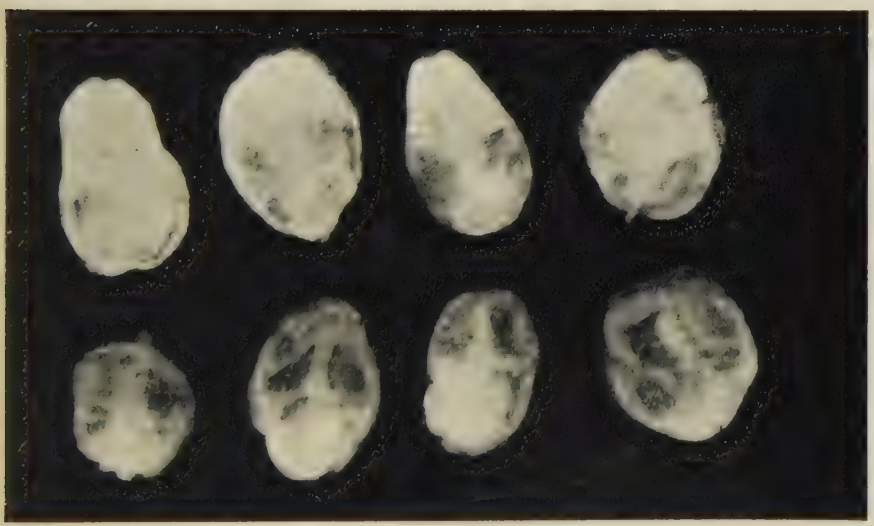


Fig. 1. Effect of copper and potassium dressings on yield of tubers. Exp. 1011, 1948. 0 = no copper supplied, 10 = copper sulphate at the rate of 10 kg per ha and 100 = copper sulphate at the rate of 100 kg per ha. (1 q = 100 kg).

*) for a description of this method see (18).

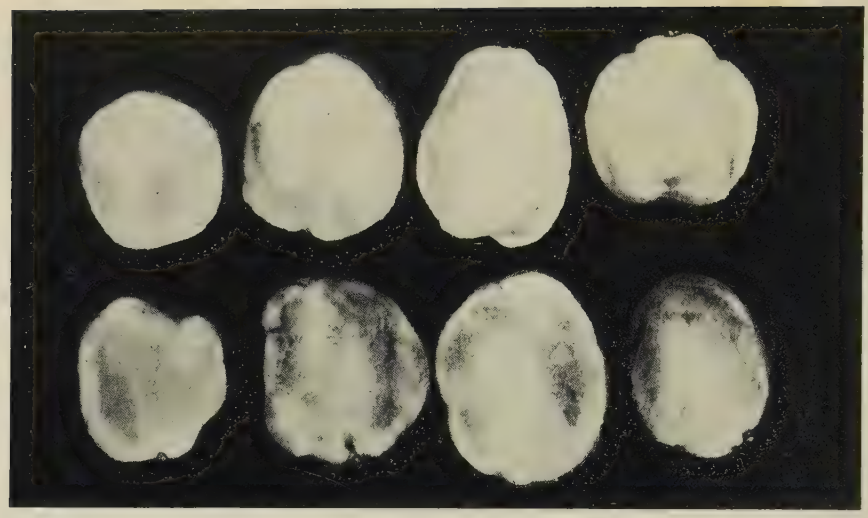


A. Attack on K-deficient Noordeling potatoes by saprophytic micro-organisms (lower row). Upper row: unaffected tubers from plants amply supplied with potassium.

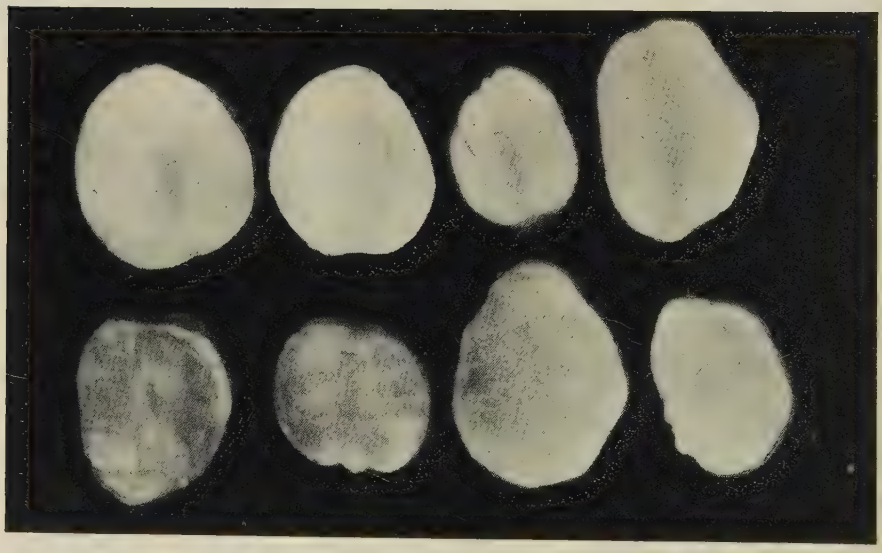


B. K-deficient Noordeling tubers (Exp. 1011, 1948). Stored from October until January, cut longitudinally and exposed to the air overnight. Upper row: tubers with a poor copper supply. Lower row: plants supplied with copper sulphate at the rate of 100 kg per ha. Yield data are given in Table 12.

Plate IV.



A



B

Plate V. Tubers from Noordeling potatoes (Exp. 1011, 1948). Stored from October until January, cut longituginally and exposed to the air overnight. A: tubers from plots dressed with potassium at the rate of 200 kg K_2O per ha. B: dressed with potassium at the rate of 400 kg K_2O per ha. Upper row of both A and B: tubers with a poor copper supply. Lower row: tubers from plants supplied with copper sulphate at the rate of 100 kg. per ha. Yield data are given in Table 12.

in this case a distinct response to the copper treatment was found as may be seen from the yield data (Fig. 1). Heavy symptoms of potassium deficiency were shown by the plants grown without a potassium dressing and by those supplied only with low amounts of this nutrient.

Potassium-deficient tubers from plots supplied with 100 kg of copper sulphate showed considerably more pronounced greyish-brown discolorations than those grown with no added copper. When the former were cut longitudinally and exposed to the air, a reddish and then black discoloration of large tissue masses developed. A close relation was to be seen between the degree of potassium-deficiency and the blackening (Plate IV and V). Without copper treatment much less blackening occurred. This was consequent on the inactivity of the tyrosinase system in the copper-deficient tubers (see Fig. 5) and not on a lower tyrosine content of the cells. This is evident from the tyrosine determinations (Table XII) together with the tyrosinase determinations to be described in the next section.

It will be seen that the copper-deficient tubers are slightly higher in tyrosine than those dressed with copper sulphate.

TABLE XII

Effect of potassium and copper treatments on yield, protein-N, soluble non-protein-N, and free-tyrosine contents of Noordeling potatoes (Exp. 1011, 1948, analysed January 1949)

Manuring *)		Yield q tubers per ha	mg in 10 g of tuber tissue			% of dry matter			Free tyrosine-N as % of soluble non-protein-N
kg $CuSO_4$ /ha	kg K_2O /ha		Protein-N	Soluble non-protein-N	Free tyrosine †	Protein-N	Soluble non-protein-N	Free tyrosine	
0	0	129	30.4	31.9	**6.1**	1.28	1.34	**0.26**	**1.50**
0	50	206	32.–	34.–	**9.3**	1.12	1.19	**0.32**	**2.08**
0	100	242	28.7	24.6	**5.4**	1.04	0.89	**0.20**	**1.74**
0	200	278	36.1	31.1	**4.4**	1.31	1.13	**0.16**	**1.10**
0	400	262	29.3	32.8	**3.4**	1.22	1.36	**0.14**	**0.80**
100	0	103	31.1	29.8	**5.7**	1.08	1.04	**0.20**	**1.49**
100	50	235	30.1	25.4	**6.3**	1.03	0.87	**0.22**	**1.95**
100	100	271	26.8	22.1	**5.1**	0.95	0.79	**0.18**	**1.76**
100	200	338	31.3	27.3	**4.–**	1.09	0.95	**0.14**	**1.14**
100	400	316	28.4	24.5	**3.6**	1.05	0.90	**0.13**	**1.12**

*) K was supplied as K_2SO_4, N at the rate of 150 kg/ha as amm. nitrate limestone, P at the rate of 200 kg P_2O_5/ha as double superphosphate and Mg at the rate of 100 kg MgO/ha as $MgSO_4$.

†) Na_2SO_3-treated samples.

e. Tyrosine content of protein from potatoes with differing mineral nutrition.

For the determination of tyrosine in potato protein use was made of tubers grown on the experimental fields mentioned above and analysed also for free tyrosine. Protein was obtained as described in section 2 (p. 63). Only the water-solube protein was used for the analyses but as it comprises approximately 90 per cent of the total, it may be assumed that the values obtained are representative of the whole protein.

In the 1946 analyses no Na_2SO_3 was added before grinding the samples. As a result the protein was somewhat brownish when hydrolysed. Furthermore in contrast to the tests of 1948 no washing with alcohol was performed.

75–100 mg of protein were hydrolysed for 24 h with 30 per cent NaOH. The excess of NaOH was then neutralized with H_2SO_4; if Na_2SO_4 was crystallizing, it was removed by centrifuging, the solution made up to volume and tyrosine determined in the usual way. Table XIII contains the results from a great number of analyses. Since the differences between the samples from the various manurial treatments were only very slight, average values of all samples from plants with heavy symptoms of potassium, magnesium, phosphate, and nitrogen deficiency respectively were calculated and compared with those from plants grown in the same field but with normal treatment. Furthermore, average values were calculated from all available Voran and Noordeling data.

TABLE XIII

Effect of certain mineral deficiencies of potato plants on the tyrosine content of the water-soluble protein in the tubers (October, 1946)

Field trial	Manurial treatment	Number of samples hydrolysed	Tyrosine as % of pure protein (16% N)
589	no potassium *)	10	5.57 ± 0.17
589	50 kg K_2O per ha*)	6	5.33 ± 0.11
589	300 kg K_2O per ha *)	9	5.55 ± 0.13
662	no magnesium *)	7	5.28 ± 0.21
662	200 kg of $MgSO_4.7H_2O$/ha *) . .	7	5.19 ± 0.19
907	no phosphate *)	9	5.48 ± 0.10
907	500 kg P_2O_5 per ha *)	12	5.11 ± 0.15
589, 662, 907	no nitrogen §)	14	4.99 ± 0.17
589, 662, 907	100 to 300 kg N per ha §) . . .	32	5.47 ± 0.05
589, 662, 907	Noordeling †)	16	5.58 ± 0.14
589, 662, 907	Voran †)	17	5.24 ± 0.09

*) Noordeling and Voran tubers with different dressings of nitrogen. Full details concerning fertilizer application, yield and contents of free tyrosine, protein, and soluble nitrogen in Tables IV, VIII and X.

†) §) Tubers differing in phosphate- and magnesium supplies.
Tubers differing in manurial treatments.

The data of Table XIII reveal that the tyrosine content of tuber protein is practically independent of the phosphate, potassium and magnesium supplies to the plants. There is some evidence that the protein of plants with an inadequate nitrogen supply is somewhat poorer in tyrosine than that of normal plants.

No varietal difference was observed.

The values found agree with those of Ross and Tottingham [29] who determined the tyrosine content of the protein from tubers blackening after cooking and from normal ones. In the former a value of 5.38% was found in the latter of 5.37%.

The figures of Table XIII are not corrected for losses sustained during hydrolysis although these undoubtedly have occurred. This may be inferred from the fact that tyrosine added before hydrolysis was recovered only to the extent of about 92%. This might mean that the above values should be multiplied by $\frac{100}{92}$. As it is uncertain that the loss of tyrosine liberated from the protein in the course of the hydrolysis is similar to that of tyrosine added before hydrolysis, this correction has not been carried out. Block and Bolling [4] found an average recovery rate of 85% and adjusted their figures using a correction factor of 1.18.

In the experiments of 1948 the recovery of tyrosine added before hydrolysis was ascertained in a great number of cases, employing different concentrations of sodium hydroxide and varying the time of hydrolysis.

The procedure followed in 1948 differed somewhat from that used in 1946. Before grinding the samples 5 cc of a 1 per cent Na_2SO_3 solution were added. Precipitation of the water-soluble protein was carried out in the manner described above. After centrifuging, the protein was washed twice with water containing a few drops of acetic acid and then three times with 96 per cent alcohol. Finally it was dried at 36°C. The nitrogen content of the protein purified in this way averaged approximately 13.6 per cent, corresponding to 85 per cent protein with 16 per cent nitrogen.

In a preliminary set of tests the influence of time of hydrolysis and concentration of NaOH solution was investigated. No difference was found between 2,5, 5 and 7.5 N NaOH for 8 hours nor between 8, 12, 16 and 24 hours hydrolysis with 7.5 N boiling NaOH. Subsequent hydrolyses therefore were carried out as follows: 50 or 100 mg of purified protein were refluxed with 20 cc of 5 N NaOH for 8 hours.

The results of these tests are shown in Table XIV.

TABLE XIV

Effect of various manurial treatments of potato plants (Noordeling) on the tyrosine content of water-soluble tuber protein (March, 1948)

Field trial	Manurial treatment	Yield of tubers q/ha	Part of tuber analysed	Number of samples hydrolysed	Tyrosine as % of pure protein (16% N)
589	0 K, 120 kg N/ha*)	104	average sample	6	6.50 ± 0.11
589	100 kg K_2O, 120 ,, ,, *)	327	bud half	6	6.46 ± 0.13
			stem half	6	6.24 ± 0.20
589	600 kg K_2O, 120 ,, ,, *)	340	bud half	6	6.67 ± 0.10
			stem half	6	6.50 ± 0.13
965	0 N, 300 kg K_2O/ha†)	201	average sample	6	6.99 ± .040
965	300 kg N, 300 ,, ,, †)	597	,, ,,	6	6.77 ± 0.15
662	0 N, 400 ,, ,, †)	107	,, ,,	6	6.42 ± 0.19
662	300 kg N, 400 ,, ,, †)	363	,, ,,	6	6.78 ± 0.11

*) Phosphate and magnesium supplied as basic dressings.
†) Supplied amply with P and Mg.

The tyrosine values found in this second set of hydrolyses are considerably higher than those found in 1946. So far, the cause of this difference is unknown. The 1948 samples were taken from tubers stored from September until March, those of 1946 shortly after harvesting the tubers. The former protein was more purified than that of 1946, while the time of hydrolysis was much shorter and the sodium hydroxide used less concentrated. Recovery of tyrosine added before hydrolysis of the 1948 samples was not greater than in 1946 viz. 83.7 ± 1.9%. The values in Table XIV have not been corrected for probable losses.

As to the effect of the mineral nutrition however, the results of 1948 agree with those of 1946. The tyrosine content of tuber protein is independent of the potassium, magnesium, nitrogen and phosphorus supplies. The small effect of nitrogen observed in 1946 was not confirmed in the 1948 samples.

Liberation of tyrosine from potato protein upon weak hydrolysis. Tottingham, Nagy and Ross[37]), while studying potatoes which blacken after cooking, found that the protein of these abnormal tubers was less firmly condensed than usual. This appeared in experiments with 3% NaOH at 37°, as well as with proteolytic enzymes. In both cases the amount of tyrosine released from K-deficient protein was greater than from normal protein.

The experiments of Tottingham *et al.* have been repeated by the author using both potassium-deficient and normal tubers. The former gave a strong melanin coloration after bruising, the latter remained white. Stem and bud ends were tested separately. 250 mg of purified protein were incubated with 70 cc of 3% NaOH at 37°C. 10 cc portions were pipetted at intervals and, after neutralisation, analysed for tyrosine. The results are given in Table XV.

TABLE XV

Effect of treatment with 3% NaOH on release of tyrosine *) from K-deficient and normal tuber protein

Protein from	mg tyrosine released from 250 mg protein (containing 12.7 mg tyrosine) after:				
	0	3½	8	27	48¼ hours
K-def. bud halves . .	0	2.7	4.1	6.9	7.5
K-def. stem halves . .	0	3.3	4.8	6.9	8.7
normal bud halves . .	0	3.3	5.6	7.5	8.9
normal stem halves . .	0	3.0	5.6	7.5	8.8

*) average values of 2 flasks.

It will be seen that protein from K-deficient tubers does not release more tyrosine upon treatment with a dilute solution of NaOH than does normal protein. The same is true whether protein from stem or bud halves are compared.

For the investigation of the enzymatic release of tyrosine from potato protein a comparison was made between tissues from K-deficient stem halves and normal bud halves. These tissues show the greatest difference as regards blackening.

10 g tissue samples were washed several times with oxygen-free distilled water, freed from adhering water between filter paper, weighed, again washed with oxygen-free water and ground under N_2. The pulp from each was transferred to a 50 cc stoppered bottle and 1 cc of toluene and 2 drops of octylalcohol were added. N_2 was then bubbled through for 5 minutes, the solution made up to volume with oxygen-free H_2O, the flasks closed and incubated at 37°. Of 10 flasks in each set two were analysed for tyrosine after 0, 2, 6, 10 and 14 days respectively.

In opposition to the results of Ross and Tottingham, which showed a considerable release of tyrosine in the course of one or two weeks, only a slight increase in free tyrosine was found by the author. As was the case when 3% NaOH was used for hydrolysis, no difference was found between the two sets of samples.

Discoloration of isolated tuber protein. It is a well-known fact that potato protein, coagulated by heat, turns brown or black upon drying. Since protein from K-deficient tubers turned brown to a much greater extent than that from normal potatoes, the question arose whether this discoloration is occasioned by the oxidation of protein-bound tyrosine by tyrosinase. According to Sizer [31] [32] such a reaction may take place.

Although the possibility of such a conversion is not denied by the author, the blackening may be explained quite as well by postulating the adsorption of oxidized free tyrosine on the protein. That this may occur was shown in the following experiment. Samples of both K-deficient and normal tubers were ground and filtered, and the filtrates exposed to the air for some hours. The former assumed a deep red colour, the latter reddened only slightly. Upon boiling the colour disappeared in both, but after cooling the solution from the K-deficient tubers became brown black, and that from the normal tubers pale grey. The coagulated protein was collected by centrifuging and washed several times with alcohol. The protein from the K-deficient potatoes was black-brown, that from the normal tubers grey. A sample of the latter, to which tyrosine had been added before grinding, gave a protein resembling that from the K-deficient tubers. Evidently oxidized tyrosine may be firmly bound by the protein, as a result of which the latter turns brown-black.

f. Localization of tyrosine and *o*-dihydric phenols in potato tubers. When slices of potassium-deficient tubers are immersed in water at 60°C for 20 minutes and then exposed to the air, reddening of the tissues outside and in the vascular ring proceeds more readily than that of the interior parts of the tuber. Since it is well-known that 3,4-dihydroxyphenylalanine, like other *o*-dihydric phenols, is oxidized more rapidly than tyrosine, it was supposed that the differences observed were due to an unequal partition of these phenols within the tubers.

To gain more information about the localization of tyrosine and *o*-diphenols in potato tubers, these compounds were determined in the layer 0–5 mm from the surface and in the tissue at a greater depth than 5 mm. The results of these preliminary tests are given in Table XVI.

TABLE XVI

Localization of free tyrosine and *o*-dihydric phenols in Noordeling potato tubers (Exp. 589, 1948, anal. March 4, 1949)

Tissue analysed	mg per 10 g of raw tissue	
	Dopa equivalent	Tyrosine
0 K, bud half, 0–0.5 cm	3.4	4.3
0 K, ,, ,, > 0.5 cm from the surface	0.7	5.9
600 kg K_2O/ha, bud half, 0–0.5 cm	1.9	2.3
600 ,, ,, ,, ,, > 0.5 cm from the surface	0.4	3.5

It will be seen that the highest value for the dopa equivalent was obtained from the exterior parts of K-deficient tubers. Tyrosine, unlike the dopa equivalent was higher in the interior parts of the tubers than in the layer 0–5 mm deep. The differences were much smaller, however, than in the case of the dopa equivalent.

In a subsequent experiment the layers 0–2, 2–6 and > 6 mm from the surface of the tubers were analysed separately. Table XVII contains the results of these analyses.

TABLE XVII

Localization of free tyrosine and dopa equivalent in K-deficient potato tubers (Noordeling, March 5, 1949)

Tissue analysed	mg per 10 g of raw tissue		% of dry matter	
	Dopa equivalent	Tyrosine	Dopa equivalent	Tyrosine
Bud half, 0–2 mm.	5.3	3.9	0.25	0.18
,, ,, , 2–6 mm.	1.4	6.4	0.07	0.31
,, ,, , > 6 mm.	0.6	6.4	0.03	0.34
Stem half, 0–2 mm.	4.6	5.8	0.20	0.25
,, ,, , 2–6 mm.	2.6	6.8	0.14	0.36
,, ,, , > 6 mm.	1.3	6.8	0.06	0.34

These data show that *o*-dihydric phenols are concentrated mainly in the exterior 2 mm of the tuber. This layer contains considerably less tyrosine than the underlying tissue. Both tyrosine and dopa equivalent are higher in the stem half than in the bud half. In a further test the periderm was analysed separately. As may be seen from Table XVIII, it contained four times as much dihyric phenol as the layer 1 mm thick immediately underlying it. In all cases both dopa equivalent and tyrosine figures were much higher in K-deficient than in normal tissue.

TABLE XVIII

Localization of free tyrosine and dopa equivalent in K-deficient and normal Noordeling potatoes (March 8 and 10, 1949)

Tissue analysed	mg per 10 g of raw tissue		% of dry matter	
	Dopa equivalent	Tyrosine	Dopa equivalent	Tyrosine
K-deficient bud half, periderm .	20.9	3.9	0.60	0.11
,, ,, ,, , 0–1 mm .	4.5	3.6	0.15	0.12
,, ,, ,, , 1–5 mm .	2.8	6.5	0.10	0.23
,, ,, ,, , > 5 mm .	1.7	7.6	0.07	0.34
Normal ,, ,, , periderm .	10.5	2.8	0.38	0.10
,, ,, ,, , 0–1 mm .	2.8	1.2	—	—
,, ,, ,, , 1–5 mm .	1.3	3.4	0.05	0.14
> 5 mm .	0.8	3.5	0.04	0.16

The results of the above experiments show that the content of dopa equivalent in the various layers of potato tubers is inversely related to the content of tyrosine. As dopa is the first oxidation product of tyrosine, it appears likely that in the external tissues part of the tyrosine has been oxidized to dopa. If this assumption be correct, it is to be expected that in Cu-deficient potatoes a smaller proportion of the tyrosine will be converted into dopa than in tubers with a normal Cu-supply. The figures of Table XIX deal with the

TABLE XIX

Effect of copper supply on the dopa equivalent and free-tyrosine contents of three tissue layers in Noordeling potatoes *) (Exp. 1011, 1948, anal. March 21, 1949).

Tissue analysed	mg per 10 g of raw tissue		% of dry matter		Dopa equiv. as % of tyrosine
	Dopa equivalent	Tyrosine	Dopa equivalent	Tyrosine	
Cu-deficient					
Bud half, 0–2 mm . .	4.3	2.2	0.24	0.12	200
,, ,, 2–5 mm . .	0.9	2.9	0.03	0.11	27
,, ,, > 5 mm . .	0.5	3.7	0.02	0.17	12
stem half, 0–2 mm . .	3.–	2.5	0.14	0.12	117
,, ,, 2–5 mm . .	0.5	4.4	0.02	0.16	12.5
,, ,, > 5 mm . .	0.3	5.7	0.01	0.25	4
Normal Cu-supply					
Bud half, 0–2 mm . .	4.8	2.–	0.26	0.11	236
,, ,, 2–5 mm . .	0.7	1.9	0.02	0.06	37
,, ,, > 5 mm . .	0.3	2.4	0.01	0.10	10
stem half, 0–2 mm . .	3.5	2.–	0.16	0.09	178
,, ,, 2–5 mm . .	0.8	3.2	0.03	0.11	27
,, ,, > 5 mm . .	0.4	4.–	0.01	0.16	7

*) tubers from plots dressed with K_2SO_4 at the rate of 400 kg K_2O/ha.

dopa equivalent and tyrosine contents of three layers in normal and Cu-deficient Noordeling tubers.

These figures reveal a higher tyrosine content in Cu-deficient tubers than in those supplied with copper sulphate, this being particularly true of the interior tissues. The dopa contents are only slightly higher in the tissues supplied with ample Cu. Expressed as percentages of the tyrosine values, however, the dopa values are considerably higher in normal tubers than in those inadequately supplied with copper.

In experiments with thin slices a fall in tyrosine content was found upon exposure to the air. In some cases a concomitant rise of the *o*-diphenol content was found, indicating a conversion of tyrosine into dopa. More details concerning these experiments will be given in a subsequent paper.

5. *Tyrosinase activity of potato tubers in relation to mineral nutrition.*

The tyrosinase activity of potato tubers was determined as described in section 2. In preliminary experiments the mixture of potato pulp and tyrosine was exposed to the air in a thin layer in wide-mouthed Erlenmeyer flasks. Since it was uncertain if under these conditions the diffusion of oxygen into the solution could keep pace with oxygen consumption, air was bubbled through the solutions in all the following experiments.

In these preliminary experiments a comparison was made of the tyrosinase activity of tubers from plants with differing potassium and nitrogen supplies. No effect of potassium was found, but tubers from plants dressed with large amounts of nitrogen had a greater tyrosinase activity than those from plants with a normal nitrogen supply. These experiments were repeated using tubers grown under various nutritional conditions. In some cases rose and heel halves of the tubers were analysed separately. The results of these experiments will be dealt with in some detail.

a. Effect of potassium supply on the tyrosinase activity.

10 g of tissue from each end of both potassium-deficient and normal Noordeling potatoes, grown on experimental field 650 in 1947, were used in this experiment. After grinding, the pulp from each sample, together with 50 mg of l-tyrosine, was made up to 150 cc and transferred to a scrubbing flask. Air was bubbled through the solution, which was incubated at 20°C, and tyrosine was determined at intervals.

The results of this experiment are represented graphically in Fig. 2. It will be seen that as regards the rose ends the tyrosinase activity in normal tubers was slightly higher than in potassium-deficient tubers. The heel ends did not show this difference. At both K-levels there was a somewhat higher activity in heel tissue than in rose tissue.

A similar test was carried out with other tubers grown on the same

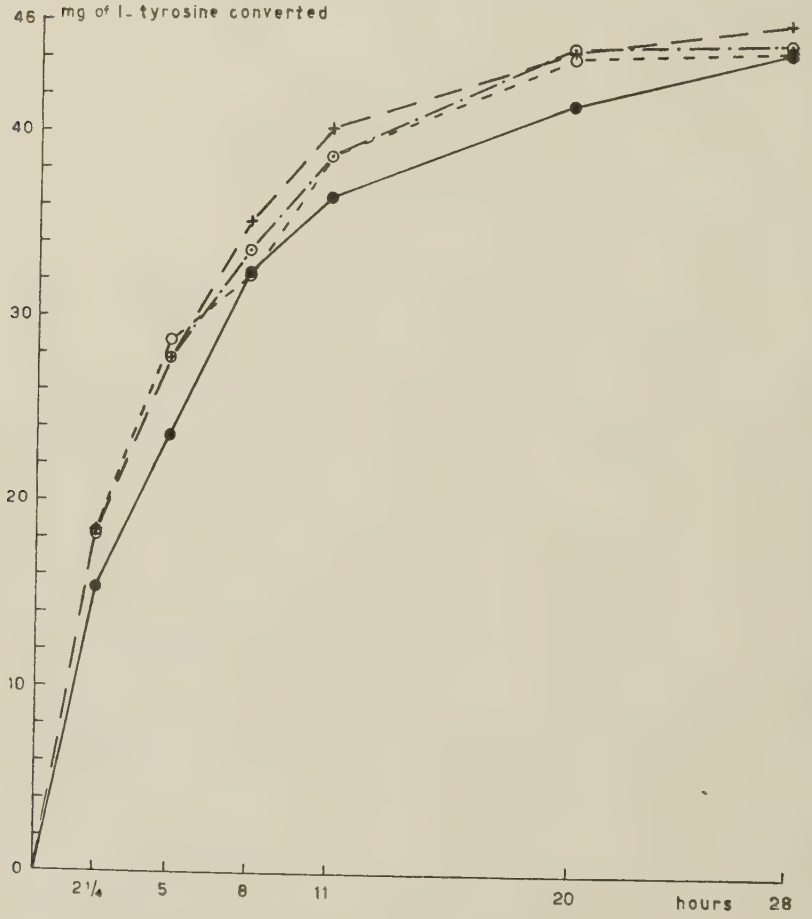


Fig. 2. Effect of the supply of potassium to potatoes on the tyrosinase activity in the tubers. Exp. 650, September 1947. ●——● rose half of tubers grown with 100 kg K_2O per ha, + – – + heel half of the same tubers, ⊙—·—⊙ rose half of tubers grown with 600 kg K_2O per ha. ○- - - ○ heel half of the same tubers. Data concerning yield and chemical composition of the tubers are given in Table V.

field, but on different plots. The results agreed with those of the first experiment in so far as the difference between heel and rose tissue was concerned. The effect of potassium supply was negligible, however.

In a third experiment, in which Noordeling potatoes from the copper-potassium experimental field 1011 were used, average tissue samples of the tubers were investigated (5 g pulp supplied with 24 mg l-tyrosine; solution made up to 100 cc; 1 cc toluene and 3 drops octylalcohol added, temperature 20°C). No effect of potassium level was found on the conversion of tyrosine.

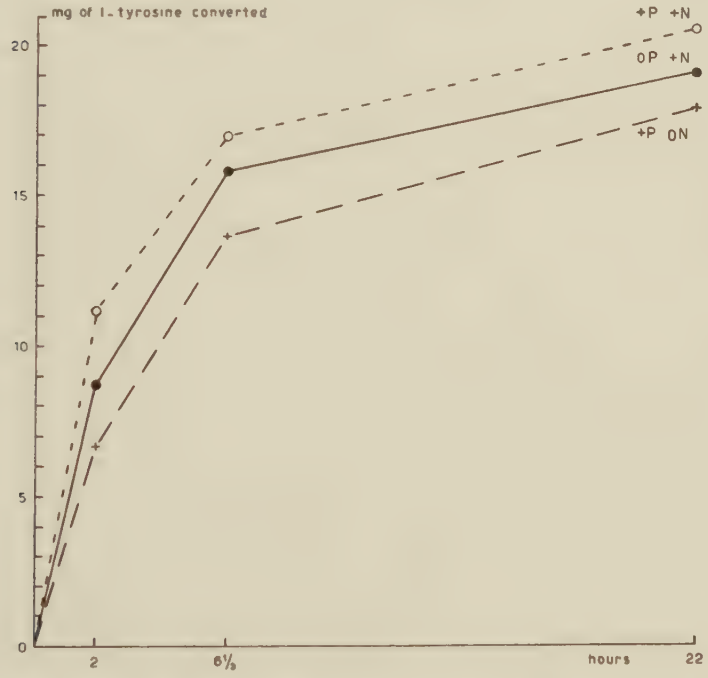


Fig. 3. Effect of the supplies of phosphorus and nitrogen to potatoes on the tyrosinase activity in the tubers. Exp. 965 January 1949. (+ P = double superphosphate at the rate of 300 kg P_2O_5 per ha, + N = $Ca(NO_3)_2$ at the rate of 300 kg N per ha).

These results demonstrate that the tyrosinase activity in potato tubers is practically independent of the potassium supply to the plants.

b. Effect of phosphorus and nitrogen supplies on the tyrosinase activity. 10 g samples of Noordeling tubers from experimental field 965, some with an inadequate and others with a normal phosphate supply, were investigated for tyrosinase activity in the manner described above. The results of this test are represented graphically in Fig. 3.

A slightly higher tyrosinase activity was found where the phosphorus supply was adequate than in phosphorus-deficient tubers. The somewhat lower values obtained from those tubers grown without a nitrogen dressing must undoubtedly be ascribed to the lower protein content of these tubers.

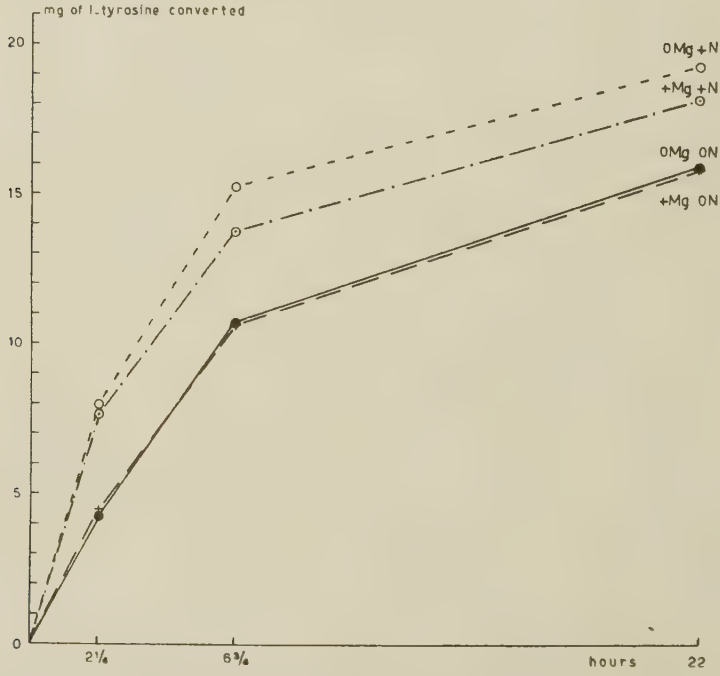


Fig. 4. Effect of the supplies of magnesium and nitrogen to potatoes on the tyrosinase activity in the tubers. Exp. 662, January 1949. + Mg = $MgSO_4$ at the rate of 200 kg per ha, + N = $(NH_4)_2SO_4$ at the rate of 300 kg N per ha.

c. Effect of magnesium and nitrogen supplies on the tyrosinase activity. For this test magnesium-deficient and normal Noordeling tubers, grown on experimental field 662 in 1948, were used. In both types 5 g samples

of tubers at two levels of nitrogen supply (poor and ample) were investigated. The results are shown in Fig. 4. It will be seen that tyrosinase activity was practically unaffected by magnesium supply. The somewhat higher values given by the magnesium-deficient tubers were probably due to their slightly higher protein contents. The relatively large differences between samples with a poor and an ample nitrogen supply must likewise be attributed to the differences in protein content.

d. Effect of copper supply on the tyrosinase activity. Since copper is the main constituent of the prosthetic group of tyrosinase, it was expected that potatoes grown on soils poor in available copper would show less tyrosinase activity than those grown on normal soils. In a preliminary experiment with Noordeling tubers grown in 1947 on a reclaimed heath soil having little available copper, a clear effect of added $CuSO_4$ was found. In 1948 the experiments were repeated with samples from field 1011, the soil of which was likewise very poor in available copper, and in potassium as well. The plan of the fertilizer treatments and yield data can be read from Fig. 1.

For each tyrosinase test an average tuber sample of 5 g was used. 24 mg of l-tyrosine, 1 cc of toluene and 3 drops of octyl alcohol were added and the solution made up to 100 cc. Air was bubbled through the solution which was incubated at 18–20°C.

After some hours' incubation a strong contrast was observed between the different solutions. Those from copper-treated potatoes had a dark reddish-brown colour whereas those from untreated plants merely showed a faint yellow shade. In the latter the tyrosinase activity was extremely low, as is shown in Fig. 5.

Potatoes dressed with copper sulphate at the rate of 10 kg per ha showed a considerably higher tyrosinase activity than those with none added. In this case the activity decreased with improving potassium supply, probably as a result of the increasing yield of dry matter lowering the copper content per unit of dry matter.

In a further set of tests, the effects of $CuSO_4$, Cu-glycinate and $FeCl_3$ on the tyrosinase activity of Cu-deficient and normal potatoes were investigated. The tubers used for this experiment were grown on plots dressed with potassium at the rate of 400 kg K_2O per ha.

Each 5 g sample of average tuber tissue was ground and the pulp transferred to a scrubbing flask, together with 40 cc of either Cu- or Fe-salt solution, 32 mg l-tyrosine dissolved in 80 cc H_2O, 2 cc toluene and 3 drops octyl alcohol. As regards the metals either 50 or 200 γ Cu as $CuSO_4$ or as Cu-glycinate, or else 0.5 or 1 mg of $FeCl_3.6H_2O$ was added per flask. After two hours' incubation the concentration of Cu-glycinate was raised from 50 to 500 γ Cu and from 200 to 800 γ Cu per flask, while the lower concentration of $FeCl_3$, was increased to 5 mg $FeCl_3.6H_2O$ per flask.

Air was bubbled through the solutions, which were incubated at 16–17°C and tyrosine was determined at intervals.

Some of the results from this experiment are represented graphically in Fig. 6. It will be seen that the Cu-deficient tubers showed no appreciable tyrosinase activity. Apparently the slight activity observed previously in these tubers had disappeared after two months'

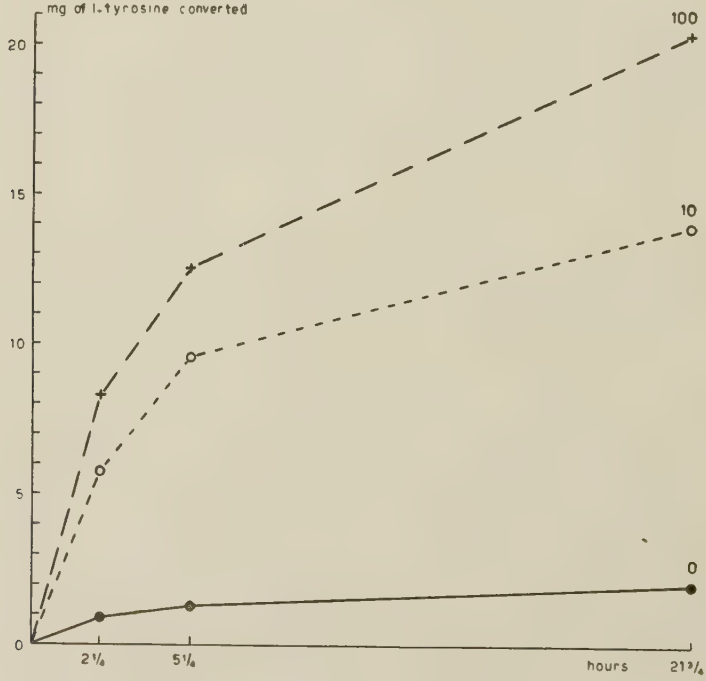


Fig. 5. Effect of the supply of copper to potatoes on the tyrosinase activity in the tubers. Exp. 1011, January 1949. 0 = no copper, 10 = copper sulphate at the rate of 10 kg per ha and 100 = copper sulphate at the rate of 100 kg per ha. In all cases potassium was supplied at the rate of 400 kg K_2O per ha. Data concerning yield and chemical composition of the tubers are given in Table XII and Fig. 1.

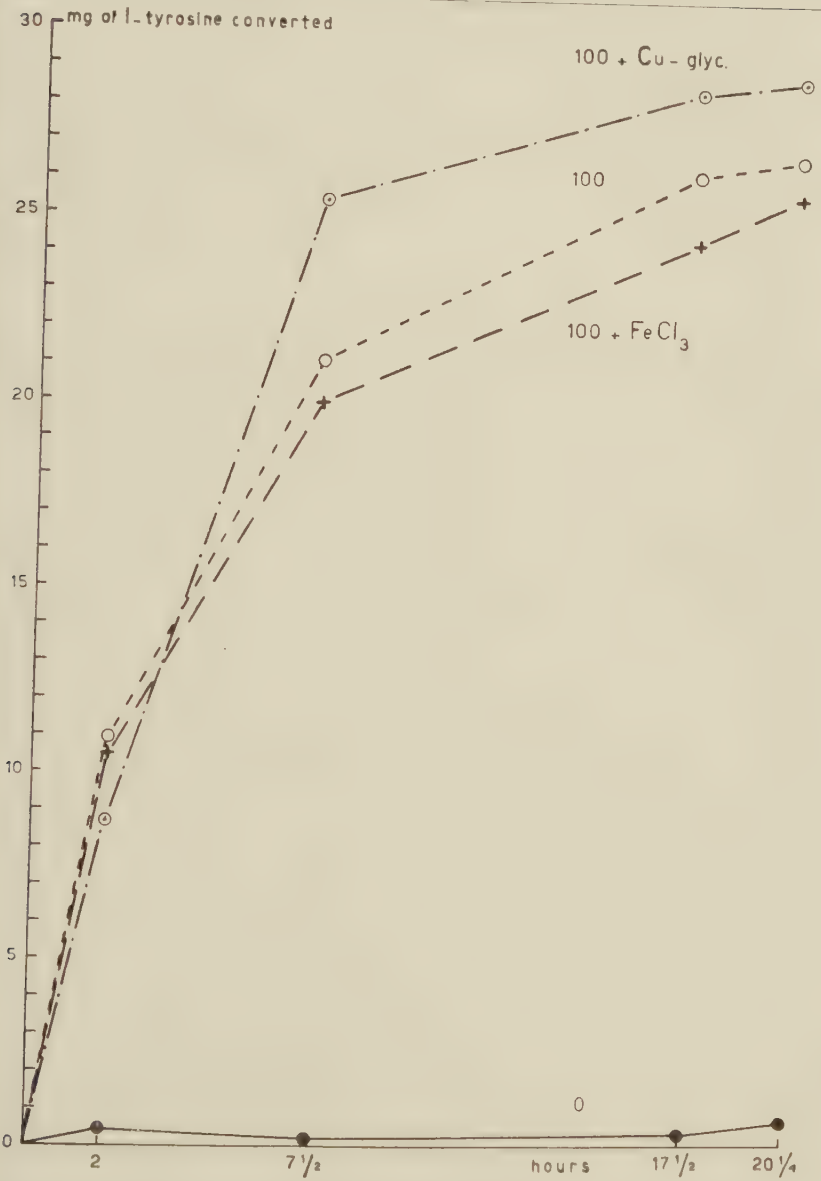


Fig. 6. Effect of Cu-glycinate and $FeCl_3$ on the tyrosinase activity of tubers grown with a poor and a normal copper supply respectively. Exp. 1011, April 1949. 0 = no copper added, 100 = supplied with copper at the rate of 100 kg $CuSO_4.5H_2O$ per ha. Cu-deficient samples supplied with Cu-glycinate or $CuSO_4$ gave values equal to or somewhat below those obtained without these additions.

storage. Neither the addition of $CuSO_4$ nor that of Cu-glycinate produced any improvement. When supplied to normal pulp, however, Cu-glycinate induced a considerable increase in tyrosinase activity. Addition of $FeCl_3$ to solutions containing pulp from normal

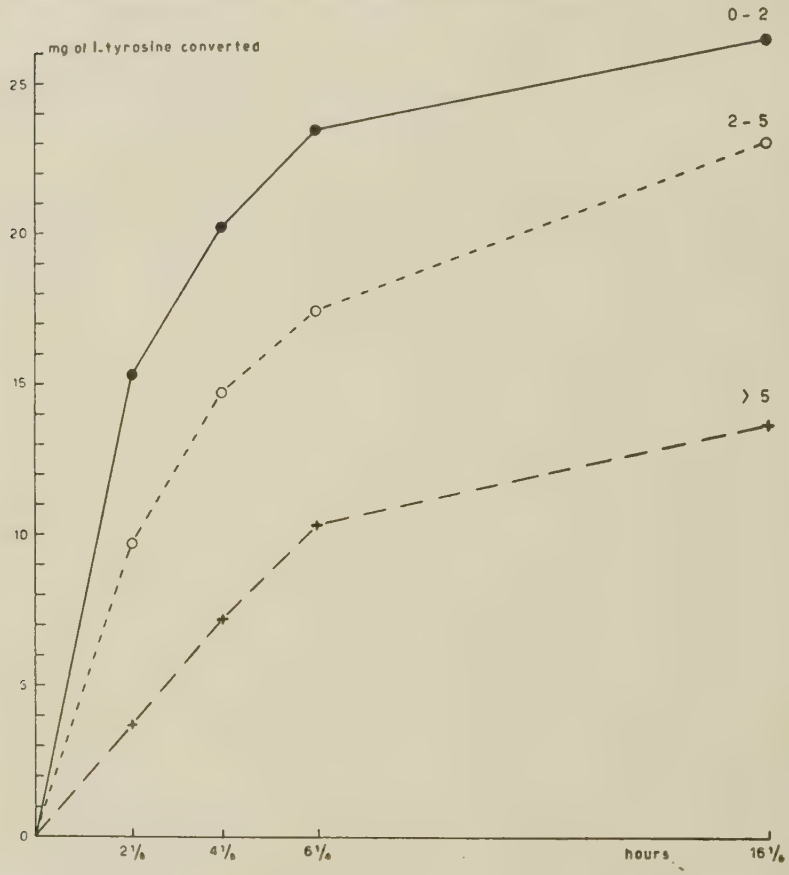


Fig. 7. Tyrosinase activity in various layers of normal potato tubers. Exp. 589, April 1949. 0 – 2, 2 – 5 and > 5 mm from the surface respectively. K-deficient tubers gave similar results, with the exception of the interior tissue which had a somewhat greater tyrosinase activity.

tubers resulted in much increased blackening. Since tyrosinase activity was not affected by the added iron (Fig. 6), it must be assumed that the intensification of blackening was caused by a reaction of Fe with one of the oxidation products of tyrosine. It is unknown whether or not under certain circumstances (high Fe content) this

reaction may play a part in the blackening of raw potatoes. In blackening after boiling the Fe-reaction may be of considerable interest, as is shown on p. 77.

As stated above, potassium-deficient tubers grown on soils poor in copper are considerably less liable to discolour than those supplied normally with copper. This in undoubtedly due to the difference in tyrosinase activity.

e. Localization of tyrosinase in potato tubers. 5 g ground tissue from the layers 0–2 (peel) and 2–5 mm and from the rest of the tubers were tested individually for tyrosinase activity in the usual way. As is shown in Fig. 7 a considerable difference in activity was found between the different tissues. The centres of the tubers contained much less tyrosinase than the tissues of the exterior layers. This difference is undoubtedly partly related to the unequal distribution of protein in potatoes. This may be concluded from the figures for total N in the various layers (Table XX).

TABLE XX

Dry matter and nitrogen content of various layers of K-deficient and normal Noordeling potatoes (Exp. 589, 1948, analysed June 4, 1949)

Potassium-deficient tubers			Normal tubers		
Layer analysed	Dry matter %	N in dry matter %	Layer analysed	Dry matter %	N in dry matter %
0–2 mm	20.5	3.98	0–2 mm	23.1	2.28
2–5 mm	25.6	2.53	2–5 mm	30.6	1.69
rest	26.5	2.28	rest	27.8	2.24

f. Effect of temperature and pH of the solution on the tyrosinase activity of potato extract. The effect of temperature on the tyrosinase activity of potato tissue was investigated in the usual way, employing Noordeling potatoes supplied with potassium, stored from October 1948 until May 1949 when the experiment was carried out. The tests took place at 20, 30, 40, 50 and 60°C respectively.

The results of this experiment are shown in fig. 8. In agreement with those obtained by Gould [10] the greatest activity of tyrosinase was found at 30°. At 40° and particularly at 50° considerable inactivation was apparent. The solutions blackened much more rapidly, however, than they did at 20 and 30° at which tempera-

tures the red colour was preserved for a much longer time. It is unknown whether this difference was caused by a much accelerated formation of melanin from the red pigment at high temperature or by the formation of the Fe-compound of one of the oxidation products of tyrosine owing to the liberation of iron.

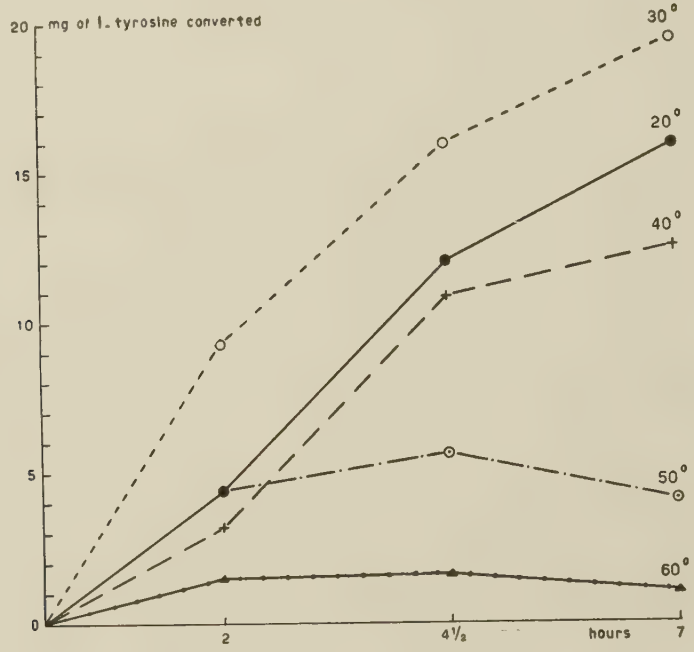


Fig. 8. Effect of temperature on the tyrosinase activity of potato pulp. Exp. 589, May 1949.

The effect of pH of the solution on the tyrosinase activity is shown in Fig. 9. It will be seen that the rate of tyrosine oxidation increased as the pH rose. The rate at which the solution blackened increased much more, however, indicating that the formation of the black pigment was affected by the pH to a greater extent than was the oxidation of tyrosine.

6. *Discussion.* Of the five elements tested (N, P, K, Mg and Cu), potassium had by far the greatest effect on the *free-tyrosine* content of potato tubers. Its effect was only partly due to the enhancement of soluble nitrogen which is typical of K-deficient plants in general. This is evident from the fact that the figures for tyrosine-N

calculated as percentages of total soluble nitrogen were highest in K-deficient plants. With increasing nitrogen supply total soluble nitrogen rose considerably but tyrosine only slightly or not at all. The accumulation of tyrosine is clearly related to some specific reaction proceeding at an enhanced rate in K-deficient potato plants.

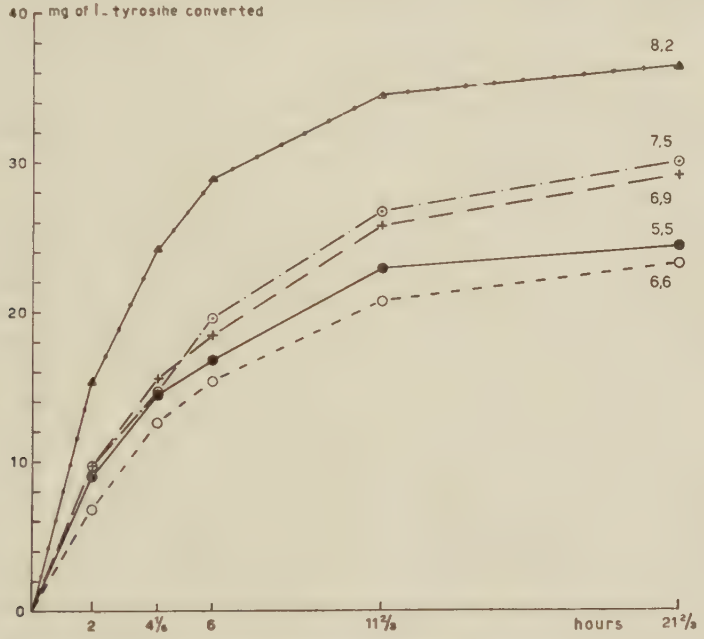


Fig. 9. Effect of pH on the tyrosinase activity of potato pulp. Exp. 589, June 1949.

More details concerning this reaction will be given in a subsequent paper.

Some evidence was obtained that magnesium deficiency has a similar but considerably smaller effect on the tyrosine content to that of potassium deficiency.

Phosphate supply did not affect the tyrosine content of potatoes.

Cu-deficient tubers again were somewhat higher in tyrosine content than normal ones. It is improbable, however, that this difference was due to increased formation of tyrosine in the Cu-deficient potatoes, but apparently was related to their low tyrosinase activity.

In spite of the high free-tyrosine content of potassium-deficient potatoes the tyrosine content of the protein did not differ from that

of normal ones nor from that of nitrogen-deficient, phosphorus-deficient and magnesium-deficient tubers. Apparently the amino-acid composition of tuber protein is independent of the concentrations of the individual free amino acids in the tissues.

Free tyrosine is distributed unequally within potato tubers. The stem halves of those investigated contained 20–40% more than the bud halves. In both halves the exterior layers were considerably poorer in tyrosine than the interior tissue. Since *o*-diphenol showed an inverse partition among the various layers, it is supposed that in the exterior tissue, part of the tyrosine is converted into 3, 4-dihydroxyphenylalanine. Tests with tubers supplied with different amounts of copper pointed in the same direction.

The *tyrosinase activity* of the tubers was found to be independent of the potassium, phosphorus and magnesium supplies to the potato plants. Tubers from nitrogen-deficient plants showed a slightly lower tyrosinase activity than those from plants supplied amply with nitrogenous fertilizers. This difference was probably due to the much higher protein content of the tubers from the latter.

In contrast to the above four nutrients, the copper supply to the plants is of great importance in determining the tyrosinase activity in the tubers. This was shown by growing potatoes on soils poor in available copper. Although the appearance of the plants was normal and the yield of tubers only slightly reduced, the tyrosinase activity was less than $^1/_{10}$ of that of tubers from the plots dressed with copper sulphate. Addition of copper sulphate or copper glycinate to pulped Cu-deficient tuber tissue supplied which l-tyrosine did not increase the activity. Apparently under these circumstances tyrosinase could not be formed. Nevertheless, when the copper salts were added to a pulp of tubers grown with a normal copper supply, the tyrosinase activity was further increased.

No difference in tyrosinase activity was found between the bud and stem halves of potato tubers. Considerable differences exist, however, with the depth of the tissues within the tubers. The tissues of the exterior layers, in which the *o*-diphenol-tyrosine ratio is much higher than in the interior parts, showed a tyrosinase activity more than twice as high as that of the latter.

Blackening of potato tubers. The blackening of raw potatoes induced by bruising should be clearly distinguished from the blackening after cooking which has been investigated and described by

numerous authors. The former is due to the enzymatic oxidation of free tyrosine or of an *o*-diphenol, presumably 3, 4-dihydroxyphenylalanine, first to a red pigment and then to the bluish-black melanin. In normal tuber tissue this conversion does not proceed, probably because of the fact that part of the tyrosine and tyrosinase are located apart and the presence of a powerful reduction system which prevents the free tyrosine or the *o*-diphenol from being oxidized when coming together with tyrosinase and oxygen. As an *in vitro* example of such a system may be mentioned a tyrosinase-dopa solution supplied with ascorbic acid. Although air may be bubbling through this solution no oxidation of dopa takes place as long as ascorbic acid is present. When all the latter substance has been oxidized, however, a rapid oxidation of dopa to red and thereafter to black pigments takes place.

In the living potato tuber, disturbance of the cell structure following injury to the tissues will allow the tyrosine oxidation to proceed. This may be demonstrated by heating tuber slices in their centres with a small flame. Oxidation of tyrosine then occurs in a ring surrounding at some distance the point of heating; in this annulus the cells are injured but the enzymes retain their activity. A similar result is obtained if the slices are either immersed for 20 minutes in water at 55–60°C or exposed to the vapour of toluene, chloroform etc. Bruising the tubers likewise causes injury to large cell complexes. In agricultural practice this latter is the most important cause of injury. Damage due to high temperature or to slight night frosts is encountered only sporadically. Although tubers with a high content of tyrosine or *o*-dihydric phenols (K-deficiency) discolour much more strongly following injury to the cells than those with a low content, the latter nevertheless show a distinct grey-black colour, provided that the copper supply to the plants was normal.

The liability of the tissues to mechanical damage depends to a large extent on the mineral nutrition. In tubers of plants amply supplied with mineral nutrients, injury to the cells results only from unusually rough handling. In general they withstand five minutes shaking in a flask without harm. In potassium-deficient tubers, however, and particularly at their stem ends, the cells are very easily damaged. A relatively slight movement of the tubers (sorting machines) causes injury to large cell complexes followed by oxidation of tyrosine and *o*-dihydric phenols to melanin. When K-deficient tubers

have been stored, they are much more easily injured than previously. Tubers from plants with a moderate potassium supply, which at harvest time showed no unusual tendency to bruise, may become rather liable to do so under storage.

Although *o*-dihydric phenols (e.g. 3,4-dihydroxyphenylalanine) are oxidized by tyrosinase much more readily than are monohydric plenols (tyrosine), it is probable that the ultimate result will be the same whether the injured cells contain tyrosine or *o*-diphenols. In the latter case reddening will appear within a minute, in the former within half an hour. In both cases blackening will occur some hours thereafter.

Of the other nutrients tested, magnesium may have some effect on blackening, for an inadequate supply may bring about a slight increase in tyrosine content.

Nitrogen supply, to which an important role has been attributed in the literature in relation to the blackening of potato tubers after boiling, was found to have only an indirect effect in the blackening following injury. When the potassium level of the soil is very low, no influence of nitrogen supply on blackening is found when it is moderate, however, the more extensive plant growth on the plots amply dressed with nitrogen may produce symptoms of K-deficiency, whereas plants grown with a low N-dressing will show no such symptoms.

Copper plays an important role in the development of black discolorations in potato tubers owing to its function in tyrosinase. Although the potato is one of those plants which need very little copper for their normal development, when grown on soils poor in this trace element, the tyrosinase activity of the tubers is very low. When, in addition to copper, potassium is lacking in these soils, the tyrosine content of the potatoes is high. In spite of this high content, blackening of bruised tissue will occur only to a small extent. Since newly reclaimed sandy and peat soils in general are poor in copper, it may be expected that after bruising potassium-deficient potatoes grown on such soils will blacken to a considerably less degree than tubers from soils well supplied with copper. It is probable that the use of copper salts as a fungicide against *Phytophtora infestans* on sandy and peat soils increases the liability to blacken. Whether this is also the case on soils already containing relatively large amounts of copper (clay soils) will be tested in the coming season. The author is not aware whether the farmers' observation that K-deficient

Noordeling potatoes grown on clay soils are more prone to blacken after bruising than tubers from sandy and peat soils, has anything to do with the copper supply. It is a well-known fact, however, that plants grown on clay soils contain considerably more copper than those from sandy or peat soils. The experiments on the effect of copper are being continued.

As to the effect of iron the following may be stated. As will be discussed below, iron is an important factor in the blackening of potatoes after boiling. It reacts with *o*-dihydric phenols to give dark blue-green pigments. Since these *o*-diphenols are also present in unboiled tubers, the question arises whether in raw tubers too the formation of Fe-compounds may be responsible for the blackening after bruising. In the present experiments such pigments were absent from the blackened potassium-deficient tubers as could easily be determined by treatment with acetic acid: melanin is resistent to such treatment, but the Fe-pigments are attacked and disappear.

When $FeCl_3$ was added to a potato extract supplied with l-tyrosine a much more rapid blackening occurred than when no Fe was added. Apparently dopa, or a later stage oxidation product reacted with the ferric ions to give a black pigment. The author therefore will not exclude the possibility that on soils extremely rich in available iron, Fe-compounds of *o*-dihydric phenols may be partly responsible for the black colour of bruised tuber areas. Investigations into this possibility are in progress in the author's laboratory.

Blackening after cooking. Although melanin formation may cause blue-black discolorations in cooked potatoes, this occurs only where enzymatic oxidation of tyrosine or *o*-diphenols to red or black oxidation products has taken place before cooking. No melanin is formed in tissues which are uncoloured before boiling. Although from a theoretical point of view it might be thought possible that during boiling, enzymatic oxidation of *o*-dihydric phenol to red pigments would take place, no such conversion was observed in K-deficient Noordeling tubers, apparently because of a lack of oxygen in the tissues. Since K-deficient tubers, after being boiled and exposed to the air, showed a bluish-green pigment, particularly at their stem ends and in the layer beneath the periderm, it must be assumed that this discoloration is due to some chemical reaction proceeding during or after the boiling. In agreement with the view advanced by J u u l it is believed that this reaction involves the formation of the ferrous

compound of an *o*-dihydric phenol, which becomes oxidized to the corresponding ferric compound upon exposure to the air. This conclusion rests on the fact that the green-blue colour developed mainly in those layers which contained most *o*-diphenol. Moreover treatment of cooked tuber slices with a 1 per cent $FeSO_4$ solution resulted in the formation of a much deeper bluish-green colour in these tissues, while if the slices were soaked first in an *o*-diphenol solution and then in a 1 per cent $FeSO_4$ solution the whole surface turned deep blue-green.

Treatment of slices from normal tubers, either before or after cooking, with a solution of an *o*-dihydric phenol caused no discoloration after exposure to the air. Since no difference was found between K-deficient and normal tubers, in content of iron soluble in dilute acetic acid, it must be concluded that the Fe-compound of *o*-dihydric phenols is produced more readily in the former than in the latter. This agrees with the observation of J u u l [12]) who believes that the pH of the tuber tissue determines the degree of discoloration. In the author's experiments, however, the difference in pH between normal and K-deficient tubers was so small that it cannot account for the difference in discoloration. Since treatment with citrate diminished considerably the degree of discoloration of cooked K-deficient tubers, it is believed that owing to the low content of citric acid in these tubers the iron reacts more easly than it does in normal tubers which according to J u u l contain much more citric acid.

In the K-deficient tubers investigated by the author the Fe-colour which developed after cooking, was much weaker than the blue-black due to melanin. It could be clearly distinguished from the latter by its greenish-blue shade. As opposed to the behaviour of the melanin colour, it disappeared upon treatment with acetic acid.

Summary

It is shown that the bluish-black discolorations found in raw potassium-deficient potato tubers are due to the enzymatic oxidation of tyrosine and *o*-dihydric phenols to melanin. Two factors are responsible for the extreme tendency of potassium-deficient tubers to blacken viz. the high content of free tyrosine and the liability of the cells to sustain injury. Owing to the operation of the latter factor tyrosine and *o*-dihydric phenols become subjected to tyrosinase activity and in consequence an irreversible oxidation to the bluish-black melanin takes place. Although *o*-dihydric phenols are converted by tyrosinase more readily than is tyrosine, the degree of blackening ultimately reached is the same with both compounds.

The blackening of potato tubers after cooking is shown to be due to melanin only in those cases in which black or red oxidation products of tyrosine or *o*-diphenols were formed before the boiling. Potassium-deficient tissues uncoloured before cooking, although very liable to bruise and high in tyrosine did not give rise to melanin formation either during or after cooking. The bluish-green pigment which developed in such tissues after cooking and exposure to the air, was found to be due to the oxidation of a ferrous compound of *o*-dihydric phenols, to the corresponding ferric compound. Tissues extremely rich in *o*-diphenols (peel, stem ends of potatoes as opposed to bud ends, K-deficient tubers in comparison with those supplied normally with potassium) discoloured to a much greater degree after cooking than those low in these compounds. No difference was found between K-deficient and normal tubers in content of iron, soluble in dilute acetic acid. In the former the iron reacted with *o*-dihydric phenols more readily than in the latter, probably owing to a lower content of citric acid.

An extensive study was made of the occurrence of free tyrosine in potato tubers having differing supplies of either potassium, nitrogen, phosphate, magnesium or copper. Potassium deficiency brought about a very high content of this amino acid. Magnesium deficiency gave a similar but much smaller increase. High dressings of nitrogen enhanced the tyrosine content only in those cases in which the K-supply was moderate, indicating that the effect of nitrogen is indirect. Copper-deficient tubers were slightly higher in tyrosine than those supplied normally with this trace element.

Stem halves of potato tubers contained about 20–40% more free tyrosine than bud halves.

Different varieties of potato showed different rates of blackening. This was found to be due to variation in both tyrosine content and the liability of the tissues to bruise.

The tyrosine content of the protein in potato tubers was found to be independent of the mineral nutrition of the plants.

No differential release of tyrosine from the protein of potassium-deficient and normal potatoes was found either upon treatment with dilute NaOH or upon proteolysis.

Free tyrosine and *o*-dihydric phenols were both found to be distributed unevenly within potato tubers. The content of tyrosine was highest in the interior parts of the tubers, but that of *o*-dihydric phenols was very low in these tissues and high in the exterior layers.

The tyrosinase activity of potato tissues was shown to be independent of the potassium, phosphate and magnesium nutrition of the plants. Nitrogen-deficient tubers had a somewhat lower tyrosinase activity, probably owing to the much lower protein content.

Potatoes grown on soils poor in copper showed a tyrosinase activity less than one tenth of that of tubers supplied normally with this element. As a result of the low tyrosinase activity, blackening of bruised tubers deficient in both potassium and copper was slight in comparison with those deficient in potassium but supplied normally with copper.

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A SIMPLE APPARATUS FOR MEASURING THE OXYGEN UPTAKE OF SOILS

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Introduction. The Haldane respirometer, described by Dixon in his standard work on manometry [1]), has been adapted for use with soils.

In this respirometer two reaction vessels carrying glass taps communicating with the atmosphere are connected by a manometer. To one vessel is attached a graduated sidearm along which some liquid (usually water or mercury) can be displaced. At the start of an experiment, the taps are opened so that the manometer fluid is level and the liquid in the graduated side-arm set to some mark. The taps are then closed. Any subsequent change in the volume of gas in one vessel not parallelled by an equivalent change in the other causes a disturbance of manometer levels. The volume of liquid that must be displaced into or out of the graduated side-arm in order to bring the manometer fluid to its original level state is then an exact measure of the differential change of gas volumes between the two vessels.

The apparatus as used for the measurement of O_2 uptake by soils is assembled with one vessel empty and the other vessel containing soil *plus* an NaOH cup for absorbing CO_2 produced by the soil. The apparatus is theoretically not sensitive to changes in temperature so long as both vessels are always kept at the same temperature. In the tropics, where diurnal changes of temperature are small, sufficient temperature stability is afforded by a box surrounding the vessels to shield them from draughts; more elaborate precautions will be necessary in temperate climates.

Apparatus and methods. The reaction vessels are the 200 ml bottles used as reservoirs in the soil percolators [2]) [3]) fitted with

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one-hole rubber bungs carrying either a glass tap alone (conventionally the LHS of the apparatus) or a glass tap *plus* a 5 ml M o h r pipette (conventionally the RHS of the apparatus). This pipette is the 'graduated side-arm' up and down which water (the displacing fluid) is moved to level the water in the manometer. The tip of the pipette is connected by approximately 0.5 metres of rubber tubing to a levelling device consisting of a 25 ml thistle funnel that can be moved up and down a burette stand by means of a clamp. The RHS of the apparatus is shown (not to scale) in Figure 1. The RHS vessel normally accomodates 50 g soil sieved 4.0 mm—1.0 mm [3]) in the centre of which stands a 5 × 1 cm cup containing 5 ml 10% NaOH and one-half of an 11 cm Whatman No. 50 filter paper (toughened paper) folded concertina-wise; this cup and its contents are the CO_2-absorption device [1]).

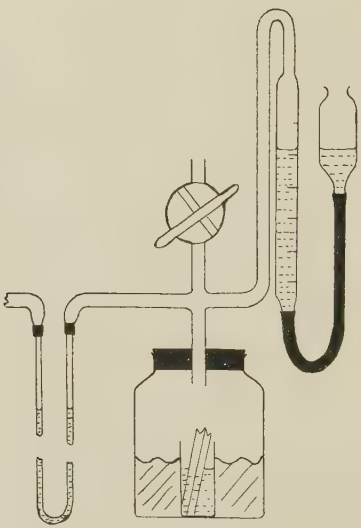

Fig. 1. The right hand vessel.

The manometer is a U-tube of 3 mm internal bore with each limb 30 cms long. It is conveniently made detachable from the vessels by means of pressure-tubing connexions; it hangs outside the draught-eliminating box in which the vessels stand, in this position the water-levels in it may easily be seen.

It is important to remember that the RHS vessel cannot, at the start of an experiment, contain more than 35 ml O_2 if the vessel is

filled with ordinary air, and that therefore an O_2 uptake of only 5 ml represents an appreciable reduction in O_2-tension in the RHS vessel. It may therefore be essential, in a long-term experiment, to disassemble the apparatus in order to admit fresh air into the RHS vessel in order to minimize the possibility of anaerobiosis therein. This procedure was adopted in the experiments described by Figures 3 and 4.

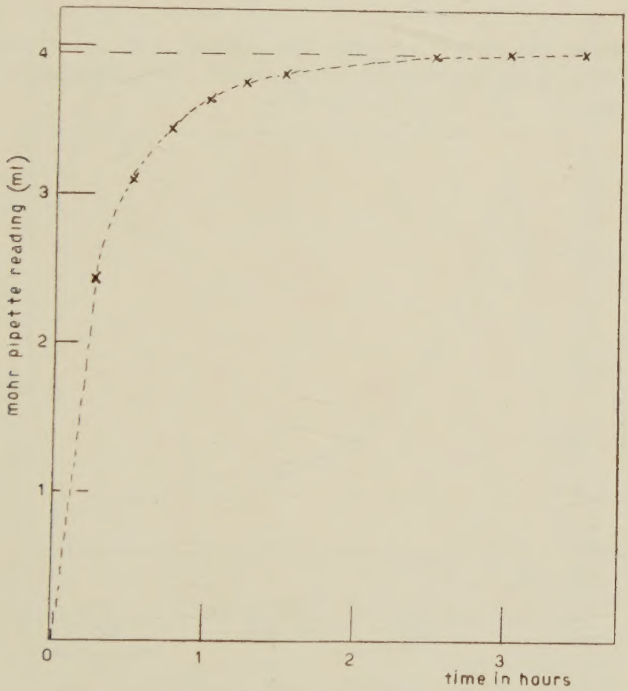

Fig. 2. Carbon dioxide absorption curve.

Results. The first experiments were a check on the efficiency of the CO_2-absorption device.

The CO_2-absorption device was placed in the *LHS* vessel which contained a solution of $NaHCO_3$ calculated to release about 4 ml CO_2 on acidification. Fitted into the bung of the LHS vessel was a 1 ml burette containing 10 N H_2SO_4. The RHS vessel was empty. With the taps open the water level in the M o h r pipette (RHS) was set at 4.95 ml; the manometer fluid was, of course, level. The taps were then closed and exactly 1.00 ml 10 N H_2SO_4 was run into the LHS vessel containing the $NaHCO_3$ solution. The M o h r pipette readings at which manometer equilibration was obtained were,

from this point on, recorded from time to time. The approach of these readings to their final theoretical value of 3.95 ml is shown in Figure 2. This Figure shows that the CO_2-absorption device works rapidly and efficiently. Experiments on the O_2-uptakes of soils were therefore begun.

The rates of O_2-uptake of a rich soil (Montserrat Clay-Silt from the San Juan district of Trinidad) and of a poor soil (Ecclesville clay from the Rio Claro district of Trinidad) were measured.

Soil samples (4.0 mm — 1.0 mm) were wetted with 20 ml water and left for a day. Subsequent O_2-uptakes, measured over corresponding 5 hour intervals each day, are shown in Figure 3.

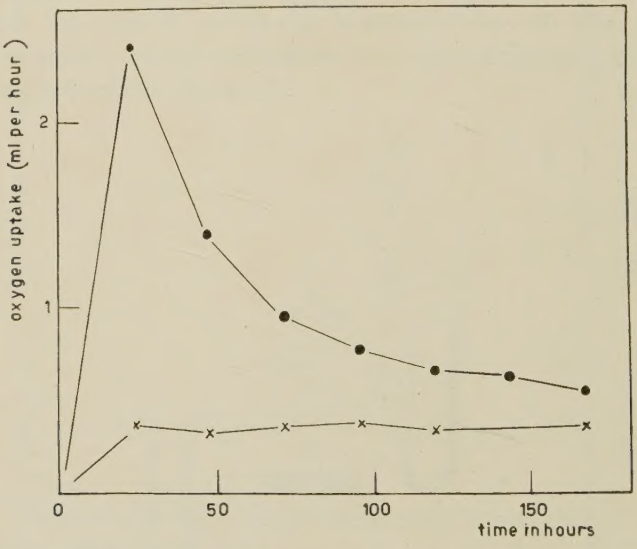

Fig. 3. Oxygen uptakes of 50 g samples of soil wetted with 20 ml water. Crosses indicate good soil; dots indicate bad soil.

The effect of glucose addition on O_2-uptake is shown in Figure 4.

Montserrat clay-silt (50 g) was wetted with 20 ml 1.0% glucose solution. The subsequent O_2-uptake of the soilglucose system is shown in Figure 4. The points in this Figure have had the 'blank' value (i.e. the rate of O_2-uptake of a similar soil-sample treated with 20 ml water) subtracted from them.

Discussion. A satisfactory apparatus for measuring the O_2-uptake of soil samples may be constructed from the reservoir assembly of the soil percolator [2]). The principle of the apparatus stems from

Haldane (quoted by Dixon) [1]) and no originality is claimed for it.

The integrated areas under the graphs of Figure 3 correspond to O_2-uptakes of 3.0 ml O_2/g soil/6 days for the poor soil and 1.0 ml O_2/g soil/6 days for the good soil. From the data given in Figure 7 of a previous paper [3]) it may be simply calculated that, when the same two soils were percolated with water, their CO_2-outputs

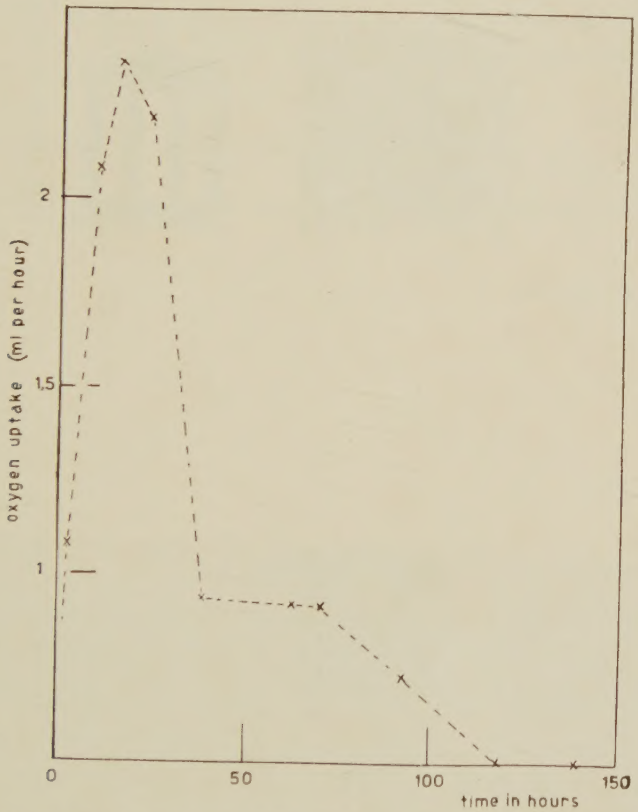

Fig. 4. Oxygen uptake curve of 50 g soil treated with 20 ml 1.0% glucose solution. Blank values for watertreated soil have been subtracted.

were 3.1 ml CO_2/g soil/6 days for the poor soil and 1.4 ml CO_2/g soil/6 days for the good soil. Thus there is good agreement between results given by the respirometer technique and those given by the percolation technique.

The integrated area under the curve of Figure 4 corresponds

to a total O_2-uptake of approximately 90 ml; complete combustion of the added glucose demanded an O_2-uptake of 160 ml. Percolation experiments [4]) have shown that only some 50—70% of the CO_2 theoretically obtainable is in fact released from glucose percolated in solution through soils. Once again, the results given by the respirometer technique agree well with those given by the percolation technique. Moreover, it is interesting to note that the shape of the O_2-uptake curve of Figure 4 is similar to the shapes of the time-temperature curves given by sucrose treated soils [5]).

Summary

1) The principle of the Haldane respirometer has been used in the measurement of O_2-uptake by soils.

2) Results obtained with the apparatus described are presented and discussed.

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